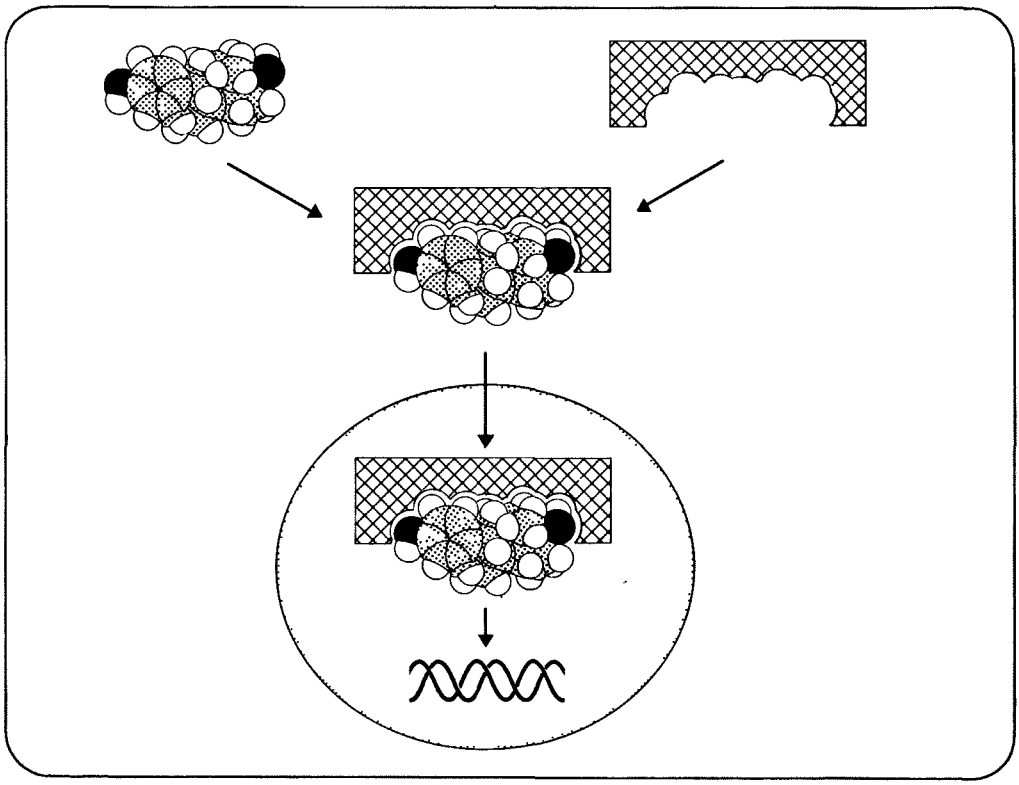
STEROID HORMONE RECEPTORS IN EXPERIMENTAL AND HUMAN MAMMARY CARCINOMA



A. J. M. KOENDERS

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PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde aan de katholieke universtiteit te NIJMEGEN, op gezag van de Rector Magnificus, Prof. Dr. P. G. A. B. Wijdeveld volgens besluit van het College van Decanen in het openbaar te verdedigen op woensdag 23 mei 1979 des namiddags te 2.00 uur precies

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Aan mijn ouders

Voor Jenny

CONTENTS

B_{NS}	nonspecific binding
B_S	specific binding
B_T	sum of specific and nonspecific binding
B/F	Bound/Free
B/F_{NS}	B/F-ratio for nonspecific binding
B/F_T	B/F-ratio for the sum of specific and nonspecific binding
BSA	bovine serum albumin
CBG	corticosteroid binding globulin
DCC	dextran-coated charcoal
DF	degrees of freedom
DMBA	7,12-dimethyl benzanthracene
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
ER	estradiol receptor
ER+	denotes the presence of ER
ER-	denotes the absence of ER
F	unbound steroid
fmoles	10^{-15} mol
g	relative centrifugal force
hr	hour(s)
K_d	equilibrium dissociation constant
M	moles per litre
min	minute(s)
N(n)	number of determinations
PgR	progesterone receptor
PgR+	denotes the presence of PgR
PgR-	denotes the absence of PgR
R	concentration of specific receptor binding sites
r	correlation coefficient
r_s	Spearman correlation coefficient
s^{-1}	$second^{-1}$

s.c.	subcutaneously
SD	standard deviation
S.E.	standard error of the mean
SHBG	sex hormone binding globulin
sq cm	length x width
T	total amount of radioactive steroid present
$t_{\frac{1}{2}}$	half life time
Tris	tris (hydroxymethyl) aminomethane
w/v	weight per volume
χ^2	chi-square test

clomiphene	:	1-[p-(β-diethylaminoethoxy)phenyl]-1,2-diphenyl-2-chloroethylene
cortisol	:	11β,17α,21-trihydroxy-4-pregnene-3,20-dione
cyproterone acetate	:	6α-chloro-17α-acetoxy-1β,2α-methylene-4,6-pregnadiene-3,20-dione
dexamethasone	:	9-fluoro-11β,17,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione
diethylstilbestrol (DES)	:	3,4-bis (4-hydroxyphenyl-3-hexane)
5α-dihydrotestosteron (DHT)	:	17β-hydroxy-5α-androstan-3-one
estradiol (E_2)	:	1,3,5 (10)-estratriene-3,17β-diol
17α-estradiol	:	1,3,5 (10)-estratriene-3,17α-diol
estriol (E_3)	:	1,3,5 (10)-estratriene-3,16α,17-triol
estrone (E_1)	:	1,3,5 (10)-estratriene-3-ol-17-one
ethynylestradiol	:	1,3,5 (10)-estratriene-3,17β-diol-17α-ethyne
hexestrol	:	3,4-bis (4-hydroxyphenyl)-hexane
medroxyprogesterone acetate	:	17α-acetoxy-6α-methyl-4-pregnene-3,20-dione
moxestrol (R 2858)	:	11β-methoxy-17-ethynyl-1,3,5 (10)-estratriene-3,17β-diol
nafoxidine (U11, 100A)	:	1-2-(p-3,4-dihydro-6-methoxy-2-phenyl-1-naphthylphenoxy) ethyl-pyrrolidine hydrochloride
Org 2058	:	16α-ethyl-21-hydroxy-19-nor-4-pregnene-3,20-dione
progesterone	:	4-pregnene-3,20-dione
R5020	:	17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione
tamoxifen (ICI 46,474)	:	trans-1-(p-β-dimethylaminoethoxy phenyl)-1,2-diphenylbut-1-ene
triamcinolone acetonide	:	9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide
U-23,469	:	cis-3-(p-1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthylphenoxy)-1,2-propanediol

CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THIS THESIS.

It has long been recognized that only one-third of all women with disseminated cancer of the breast respond to endocrine therapy (surgical ablation, hormone additive therapy or the administration of hormone antagonists) (Hayward, 1970; Stoll, 1970). A method of predicting a priori a breast cancer patient's response to endocrine therapy would greatly enhance the usefulness of endocrine therapy since 2 out of 3 women would be spared unnecessary endocrine measures (Jensen et al., 1967). In addition 2 out of 3 patients could receive alternative types of therapy earlier, which may be more beneficial. A link between the hormone responsiveness of human breast cancer and the ability of such tumors to accumulate estrogen was discovered by Folca (1961). These investigators demonstrated that, following injection of tritiated hexestrol, patients with breast cancer who responded favourably to adrenalectomy accumulated more radioactivity in their tumors than those patients who showed no improvement.

During the past decade considerable insight has been gained concerning the mechanisms by which estradiol initiates specific phenotypic effects in target tissues. There is ample evidence that the initial step in estradiol action is the binding of the hormone to specific cytoplasmic receptor proteins. Estradiol receptor proteins are present in target tissues such as uterus, vagina, anterior pituitary and mammary gland but are absent in non-target tissues. The estradiol receptor is highly steroid specific and the estradiol receptor interaction is characterized by a very high affinity ($K_d \sim 10^{-10}$M). In the presence of estradiol, the receptor undergoes a temperature-promoted dimerisation with a second similar or dissimilar monomer to form the activated receptor complex (receptor transformation). The transformed complex migrates or translocates to the nucleus (receptor translocation), where it binds to acceptor sites in the chromatin. This interaction initiates a chain of events resulting in gene transcription and altered protein synthesis. For detailed information regarding the mechanism of action of steroid hormones, the reader is referred to recent reviews on this subject (Jensen and DeSombre, 1973; Gorski and Gannon, 1976; O'Malley and Birnbaumer, 1977; Yamomoto and Alberts, 1976).

Based on the premise that the estradiol receptor is a necessary requirement for estradiol stimulation of tissue growth, Jensen et al. (1967) suggested that by measuring the uptake of tritiated estradiol by tumor tissue in vitro it might be possible to predict which patients will respond to endocrine treatment. It is now generally acknowledged that the estradiol receptor is useful in selecting patients for endocrine therapy. In several series about 60-75% of all breast cancers have found to contain measurable amounts of estradiol receptor. The collected data from a dozen centers around the world showed that 55-60% of the patients with estradiol receptor-positive tumors and only 8% of the patients with estradiol receptor-negative tumors responded to endocrine therapy (McGuire, Carbone and Vollmer, 1975). The estradiol receptor assay is therefore most useful in excluding patients with estradiol receptor-negative tumors from all types of endocrine therapy. However, about 40% of the patients with estradiol receptor-positive tumors fail to respond to endocrine therapy.

Recently it has been emphasized that in endocrine unresponsive tumors, which contain cytoplasmic estradiol receptors, there may be biochemical lesions distal to the initial binding step (Shyamala, 1972). Therefore, it may be preferable to measure an end product of estrogen action rather than the initial binding step. Because in normal estrogen target tissues the synthesis of progesterone receptor is dependent on and stimulated by estradiol, McGuire et al. (1975) hypothesized that the progesterone receptor could serve as a reliable marker of endocrine responsiveness of breast tumors. In other words, the presence of progesterone receptor in estradiol receptor-positive tumors would indicate that the tumors remained endocrine responsive. On the other hand, the response rate to endocrine therapy would be very low in tumors which contain estradiol receptor but no progesterone receptor. Moreover, progesterone receptor should not be found in estradiol receptor-negative tumors. This attractive hypothesis regarding the progesterone receptor as a reliable marker of endocrine responsiveness of breast tumors is currently under investigation in this as well as in various other laboratories.

In Chapter 2, the dextran-coated charcoal assays developed for the measurement of estradiol and progesterone receptors are evaluated with special emphasis on reproducibility, specificity and the criteria used to classify tumors as receptor-positive or receptor-negative. As discussed earlier, patients will be excluded from all types of endocrine therapy on the basis of an estradiol receptor-negative tumor classification.

Therefore experimental conditions (e.g. cytosol dilution, cytosol protein concentration and interference of plasma protein contamination), which might have an influence on the classification of a tumor as receptor-positive or receptor-negative were carefully examined. In the literature there is only very limited information available regarding the intra- and inter-assay variation of the assays employed within a particular laboratory or regarding the assay variation between different laboratories. In this laboratory a tissue preparation was developed, which was used to study the inter-assay variation of the estradiol and progesterone receptor determinations and in addition seems suitable for inter-laboratory control studies.

The experimental DMBA-tumor model has been most widely used in the study of mechanisms underlying hormone dependency of breast cancer (Leung, 1978). Tumor induction is carried out in female Sprague-Dawley rats by a single intragastric feeding of 20 mg DMBA at the critical age of 50 days (Huggins et al., 1961). Mammary tumors induced by DMBA administration are, just as human breast tumors, of ductal origin and have the histopathological features of adenocarcinomas (Sinha and Dao, 1975). About 60-90% of the tumors are regarded as hormone-dependent, since they regress after endocrine ablations, additive hormone treatments or the administration of hormone antagonists (Huggins et al., 1961; Pearson et al., 1972; Leung, 1978). This DMBA-tumor model has proved to be of value: a) for validation of new approaches and assays for the detection of hormone dependency. In fact King et al. (1965) and Mobbs (1966) used this animal model to demonstrate that hormone-dependent mammary tumors show a specific uptake of $^{3}H\text{-}E_2$ in vivo, similar to that seen in normal estrogen target tissues. Moreover Jensen et al. (1967) reported for the first time that tissue slices of ovarian-dependent tumors showed a greater incorporation of $^{3}H\text{-}E_2$ than ovarian-independent tumors. b) for the selection and screening of new compounds with potential antitumor activity. For example the effects of antiestrogens and antiprolactin drugs on tumor growth have first been evaluated in this experimental tumor model before these compounds were subjected to clinical trials in advanced breast cancer (Heuson et al., 1976). c) for improving our knowledge of control mechanisms regulating tumor growth (Leung, 1978). Estrogens and prolactin have shown to be of importance for the development and growth of DMBA-tumors (Bradley et al., 1976; Manni et al., 1977; Leung, 1978). Estrogen exerts its influence on tumor growth by stimulating prolactin secretion by the pituitary and in addition estrogen and prolactin act

synergistically and directly at the tumor level. Studies with prolactin inhibitors suggest that prolactin may not play as important a role in human breast cancer as in this animal model (European Breast Cancer Group, 1972). One of the difficulties with this DMBA-tumor model is that established tumors may undergo a spontaneous regression independent of the hormonal state of the animal, and hormone dependency may change with the age of the tumors (Young and Cowan, 1963; Griswald and Green, 1970). However, its major drawback is that tumors may invade local tissues but rarely, if ever, metastasize (Dao, 1964).

In chapters 3 and 4 the results of studies performed with this tumor model are described, the objectives of which were: 1. to investigate whether responsiveness of DMBA-tumors to endocrine treatment (ovariectomy, estradiol administration and treatment with the antiestrogen tamoxifen) is related to quantitative ER levels; 2. to study whether the synthesis of the progesterone receptor in these malignant tumor cells, as in normal target tissues, is dependent on and stimulated by estradiol; and 3. to evaluate the hypothesis that the progesterone receptor is a reliable marker of endocrine responsiveness of breast tumors.

In chapter 5 the presence of estradiol and progesterone receptors in primary and metastatic human breast tumors has been studied. In particular conditions (such as menopausal status, contraceptive steroids, endocrine therapy and the stage of the disease) which might be of influence on the interpretation of these receptor data were analysed.

The method evaluated in this thesis has been used in a collaborative clinical study described in the thesis of L.V.A. Beex (1979). This clinical study relates the estradiol receptor activity to results of treatment and to characteristics of the morbid history of the patients.

CHAPTER 2

EVALUATION OF THE METHOD DEVELOPED FOR MEASUREMENT OF ESTRADIOL AND PROGESTERONE RECEPTOR BINDING SITES.

2.1 Introduction

During the past ten years a large number of procedures for the measurement of cytoplasmic estradiol and progesterone receptors have been described. All of these methods depend on occupation of the available binding sites by added labeled hormone, a binding which depends on the reversible formation of complexes between receptor proteins and radioactive steroid molecules.

A variety of techniques are employed to separate the receptor-hormone complex from unbound hormone. The methods most commonly used are sucrose gradient centrifugation (Jensen et al., 1971; Wittliff, 1974), agargel electrophoresis (Wagner, 1978; Korstens, 1972) and dextran-coated charcoal separation (Korenman and Dukes, 1970; Feherty et al., 1971). Each of these methods has its own particular advantages and disadvantages (McGuire, Carbone and Vollmer, 1975) but in all of them the equilibrium between the reactants is ultimately lost, as one of the components is removed. Because of these non-equilibrium conditions it is highly desirable to determine the number of binding sites by Scatchard analysis (Scatchard, 1949). An additional advantage of such an analysis is the possibility to monitor the specificity of each determination by calculating the apparent equilibrium dissociation constant. The dextran-coated charcoal assay is most suited for performing Scatchard analysis. Its major disadvantage is however the possible interference of plasma proteins with steroid binding to receptor binding sites.

In the present study dextran-coated charcoal assays for the estradiol and progesterone receptors have been developed on a micro scale, allowing construction of a six-dose Scatchard plot even when relatively small amounts of tissue are available for analysis. As the radioactive ligand for the assay of ER, ^{3}H-estradiol was used. For the assay of PgR a radioactive synthetic progestin ^{3}H-R5020 was employed. As compared with progesterone itself, this compound hardly displays affinity towards corticosteroid binding globulin and dissociates less rapidly from progesterone receptor binding sites (Raynaud, 1977).

An extensive validation of the receptor assays developed, is presented in this chapter.

2.2 Procedure

2.2.1 Collection and storage of biopsy specimens

Human tissue specimens were obtained through the cooperation from surgeons, internists and pathologists of the Canisius/Wilhelmina Hospital (Nijmegen) and the Radboud Hospital (Nijmegen). As far as macroscopically possible excised breast cancer tissue specimens were freed from fat, connective and necrotic tissue by the pathologist. A representative part was used for histological examination, the remaining tissue was immediately deep frozen (dry ice) and stored at -70^{o}C. Within two weeks the tissue was transferred to our laboratory and stored in liquid nitrogen. Almost all metastatic biopsy specimens, after removal of a representative part for histological examination, were immediately placed on dry ice and within a few hours stored in liquid nitrogen. All biopsies were analysed within 2 to 3 weeks after arrival at the laboratory. The excision and storage of DMBA-tumor tissue will be described in chapter 3.

2.2.2 Preparation of cytosols

Frozen tissue was first pulverized in a stainless steel mortar immersed in liquid nitrogen. Thereafter the small pieces were vibrated for 45 sec at maximum frequency to a fine powder by means of a microdismembrator (Braun, Melsungen, Germany). The frozen tissue powder was transferred to a TenBroeck homogenizer and left to thaw on an ice bath. All subsequent steps were performed at 0 to 4^{o}C. The tissue powder was homogenised in an appropriate volume of cold buffer. When enough tissue was available, 250 mg of tissue powder was weighed and 1.00 ml of buffer added, obtaining a w/v-ratio of 1/4. The following buffers were used:

TED-buffer: Tris-HCl 10 mM, EDTA 1.5 mM, DTT 0.5 mM, pH 7.4 for the determination of the estradiol receptor (McGuire at al., 1975).

TEG-buffer: Tris-HCl 10 mM, EDTA 1.5 mM, α-monothioglycerol 10 mM, 10% glycerol (vol/vol) pH 7.4 for the determination of the progesterone receptor (Rao et al., 1974).

When less than 250 mg tumor tissue was available a smaller w/v-ratio was chosen as for technical reasons at least 0.8 ml buffer was added to the prepared tissue powder. The homogenate was centrifuged at 105,000 g for 60 min to prepare the cytosol, which was removed by pipetting. Care was taken to avoid contamination from the upper lipid layer. The prepared cytosol was always analysed without delay.

2.2.3 Dextran-coated charcoal assay

All receptor assays were performed in microtiterplates (v shape, 96 holes and maximum volume 250 µl) (Katzenellenbogen et al., 1973). Fig. 2.1 gives an outline of the procedure for the determination of cytoplasmic estradiol and progesterone receptor activity. To hole 1 and 2 buffer instead of cytosol was added serving as a control for the removal of free steroid by the dextran-coated charcoal (DCC) suspension. With different batches of tracer the charcoal blank (blank) varied between 0.5 and 2.0% of the total amount of radioactive steroid (T) present. Hole 3 and 4 were used for determination of T. In a routine assay 50 µl cytosol aliquots were incubated with six concentrations of ^{3}H-ligand ranging between 10^{-9} and 8.10^{-9}M for ^{3}H-E_2 and between 10^{-9} and 10^{-8}M for ^{3}H-R5020. The sum of specific and nonspecific binding (B_T) was determined in duplicate (hole 5 and 6). The binding in the presence of 10^{-6}M nonradioactive ligand, the nonspecific binding (B_{NS}), was determined at each ^{3}H-ligand concentration (hole 7). The cytosol was preincubated with nonradioactive ligand 15 min prior to the addition of radioactive ligand. Radioactive ligand solutions were prepared shortly before use from a stock solution (10^{-7}M) stored at -20°C in ethanol. After evaporation of the ethanol the ^{3}H-ligand was dissolved in buffer containing 0.01% BSA. After incubation for 16-20 hours at 0 - 4°C, the unbound steroid was removed by adding 0.1 ml DCC-suspension. This suspension (0.25% Norit A and 0.025% dextran T_{70} in TED-buffer) was prepared and slowly stirred overnight at 0 - 4°C before each series of determinations. Eight 100 µl DCC-aliquots were added simultaneously by means of a multichannel pipettor. The plates were shaken for 10 min at 0 - 4°C on a mechanical shaker and thereafter centrifuged for 20 min at 1000 g to sediment the charcoal. Aliquots (0.1 ml) of the supernatants were transferred to counting vials containing 3.0 ml scintillation fluid. The radioactivity was counted with an efficiency of 46%.

Fig. 2.1 Procedure for the assay of estradiol and progesterone receptors.

Hole no.[a]	1-2	3-4	5-6	7
Buffer	55 µl	155 µl	5 µl	-
Unlabeled steroid (10^{-6}M)	-	-		5 µl
Cytosol	-	-	50 µl	50 µl
Preincubation 15 min at 0 - 4°C				
^{3}H-steroid[b]	5 µl	5 µl	5 µl	5 µl
Incubation 16 - 20 hr at 0 - 4°C				
Dextran-coated charcoal	100 µl	-	100 µl	100 µl

Vibrate the plates for 10 min at 0 - 4°C and centrifuge at 1,000 g for 20 min; take 100 µl aliquots for radioactivity counting.

[a]For each of the six different ^{3}H-steroid concentrations

[b] ^{3}H-E_2: 1 - 8 nM and ^{3}H-R5020 : 1 - 10 nM.

In those cases in which less than 200 mg tissue was available the cytosol volume obtained from 0.8 ml homogenate was in the order of 0.6 ml. Three 50 µl cytosol aliquots were incubated with ^{3}H-ligand (1.0, 2.0 and 8.0 nM), whereas the nonspecific binding was determined only for the two highest ^{3}H-ligand concentrations. Thus 600 µl cytosol was enough to determine the concentration of estradiol as well as progesterone receptor binding sites by a three point Scatchard analysis.

2.2.4 Calculation of Receptor binding data

All corrections and calculations were carried out by a programmable Wang 600 calculator. For each ^{3}H-ligand concentration the corresponding charcoal blank was subtracted from the measured amounts of bound radioactivity.

These corrected values (B_T - blank = B'_T; B_{NS} - blank = B'_{NS}) were subtracted from the total amount of radioactivity present (T, hole 3 and 4), giving the corresponding amounts of unbound steroid ($T - B'_T = F_T$ and $T - B'_{NS} = F_{NS}$). The specific receptor binding (B_S) was calculated according to the following equation (Chamness and McGuire, 1975):

$$B_S = B'_T - F_T \times B'_{NS}/F_{NS}$$

It may be noted that the amount of nonspecifically bound ligand was calculated at the same free ligand concentration with which receptor bound ligand was in equilibrium (Rosenthal, 1967). This equation can only be used if the B'_{NS}/F_{NS}-ratio is independent from the steroid concentration present. If this ratio was not constant the data were corrected graphically according to the method described by Rosenthal (1967). The calculated B_S-values were plotted against the corresponding B_S/F_T ratio's giving the Scatchard plot. After calculation the line of best fit (least squares), the number of specific receptor binding sites (R) was determined from the intercept on the abscissa. The equilibrium dissociation constant (K_d) was calculated from the reverse of the slope of the straight line. The concentration of receptor binding sites was expressed as fmoles/mg protein. The protein content of each cytosol was determined by the method of Lowry (Lowry et al., 1951) using BSA as the standard.

2.3 Evaluation of receptor binding data

2.3.1 Scatchard analysis

The specificity of each determination was monitored by performing Scatchard analysis. Various types of Scatchard plots were obtained. These types as well as the frequencies of their occurrence are shown in Fig. 2.2. In a vast majority of the tumors, classified as receptor-positive, linear plots (Fig. 2.2a) were observed. The remaining tumors, scored as receptor-positive, showed linear portions in their plots from which low K_d-values could be calculated (Fig. 2.2b-e). The plot shown in Fig. 2.2b could reflect ^{3}H-ligand binding to at least two different binding proteins e.g. ^{3}H-E_2 bound to its receptor and to Sex Hormone Binding Globuline (2.5.2). Scatchard plot type 2.2c could result from an overestimation of nonspecific binding.

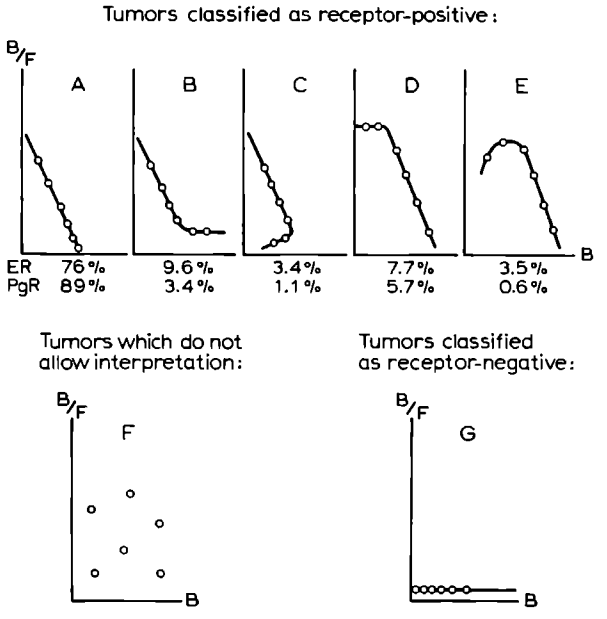


Fig. 2.2 Various types of Scatchard plots observed when human breast tumor cytosols were analysed for the presence of estradiol and progesterone receptors.

Similar Scatchard plots were obtained, when the nonspecific binding (B_{NS}) was determined in the presence of antiestrogens such as clomiphene and nafoxidine (5.10^{-5}M). These estrogen antagonists do not effectively compete for all estradiol receptor binding sites, because of the relatively low affinity compared to estradiol itself, resulting in an overestimation of nonspecific binding. The type of Scatchard plot in Fig. 2.2d was almost exclusively observed in tumor cytosols containing high receptor values. In these cases the B/F ratios for the lowest ^{3}H-ligand concentrations were greater than four. Several investigators have observed the type of non-linear Scatchard plot shown in Fig. 2.2e. It has been reported that the curvature at low ^{3}H-ligand concentrations is perhaps due to 1. instability of unoccupied receptor sites (Chamness and McGuire, 1975) 2. positive cooperativity

(Erdos et al., 1971) 3. inaccurate separation of free and bound ligand (Boeynaems and Dumont, 1975). In about 2% of all breast tumors analysed it was observed that the points do not fit a straight line, but were more or less scattered (Fig. 2.2f). Especially tumor cytosols with receptor values in the order of the lower limit of detection showed such Scatchard plots. It is obvious that the receptor concentration and the K_d-value can not be calculated from such data and these tumors could therefore not be classified and were excluded from the present study. Finally Fig. 2.2g gives an example of a receptor-negative tumor.

An acknowledged advantage of Scatchard analysis is that an estimate of the dissociation constant (K_d) is readily obtained, which should fall within a predicted range (McGuire, Carbone and Vollmer, 1975). The K_d-values, calculated from Scatchard plots with linear portions, ranged between 0.02 and 9.0 nM for the estradiol receptor (0.60 $\pm$ 0.65, n=270) and between 0.06 and 10.0 nM for the progesterone receptor (1.0 $\pm$ 0.85, n=190). Fig. 2.3 shows the frequency distribution of K_d-values for ER and PgR determinations in human breast tumor cytosols. No normal distribution of K_d-values was obtained. Therefore a discrimination between receptor-positive and receptor-negative tumors on the basis of the K_d-value could not be reached on usual statistical terms. However K_d-values above 4 nM for ER and PgR do not belong to a continuous distribution. Therefore arbitrarily tumors with K_d-values above 4 nM were scored as receptor-negative. According to this strategy 2 tumors and 8 tumors, that have been analysed for the presence of ER and PgR respectively (1-2% of all tumors) were classified as receptor-negative although their plots appeared to have a linear portion.

2.3.2 Lower limit of sensitivity

The lower limit of sensitivity was only roughly approached because, in this type of assay, not only the precision at low concentration determines this limit but additionally the arbitrarily chosen lower cutoff value for the K_d of the Scatchard plot plays a role.

Another point of relevance is the often small size of the sample, which has to be analysed. The cytosol volume required to perform a complete Scatchard analysis with 6 different concentrations of ^{3}H-ligand is 0.9 ml. This volume is obtained from 250 mg tumor tissue, when the w/v-ratio is 1/4.

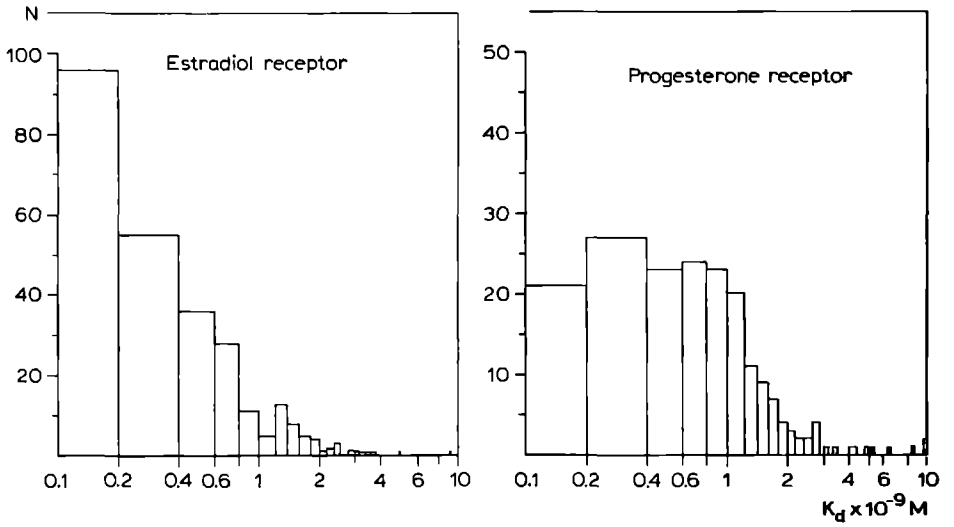


Fig. 2.3 Frequency distribution of K_d-values for estradiol and progesterone receptor determination in human breast tumor cytosols.

In 45% of the primary and 75% of the metastatic breast tumors analysed the available amount of tumor biopsy specimen was less than 250 mg. In these cases the w/v-ratio was smaller, resulting in a lower cytosol protein content. Therefore the relation between protein content and the receptor binding capacity was studied. The results of two representative experiments, performed with calf uterus cytosol, are collected in Tables 2.1 and 2.2. The undiluted uterine cytosol (w/v-ratio, 1/4) revealed an ER of 360 fmoles/mg protein. It can be seen from table 2.1 that a small decline of the concentration of binding sites was observed at a 4-fold cytosol dilution, with a corresponding protein content of 1.4 mg/ml cytosol. Nevertheless at a 16-fold dilution (0.35 mg protein/ml cytosol) the value measured was still 83% of the receptor activity measured in the undiluted cytosol with a w/v-ratio of 1/4. The actual amount of ER measured in this 16-fold diluted cytosol was 105 fmoles/ml cytosol.

Table 2.1 Influence of dilution of calf uterus cytosol on the measurement of ER binding sites.

Dilution factor	mg protein / ml cytosol	ER (fmoles/ml cytosol)	ER (fmoles/mg protein)
0	5.6	2020	360
1.3	4.2	1470	350
2	2.8	980	350
4	1.4	460	330
8	0.70	220	315
16	0.35	105	300

Pulverized calf uterus tissue was homogenised with 4 volumes/g of tissue. The cytosol, prepared by centrifugation of the homogenate (w/v-ratio, 1/4), was diluted with different volumes of buffer and at each dilution incubated with 6 concentrations of ^{3}H-E_2, allowing Scatchard analysis.

Table 2.2 Influence of dilution of calf uterus cytosol on the measurement of PgR binding sites.

Dilution factor	mg protein / ml cytosol	PgR (fmoles/ml cytosol)	PgR (fmoles/mg protein)
0	2.6	1740	670
1.3	2.0	1340	670
1.8	1.4	880	630
2.25	1.1	760	690
3	0.85	600	710
9	0.30	200	670

Calf uterus tissue was pulverized and homogenised in 4.0 ml buffer/g of tissue. The homogenate was centrifuged to obtain the cytosol. The cytosol (w/v-ratio, 1/4) was diluted with different volumes of buffer and at each dilution incubated with 6 concentrations of ^{3}H-R5020 allowing, Scatchard analysis.

Table 2.3 Influence of dilution of human breast tumor cytosol on the measurement of ER binding sites.

Dilution factor	mg protein / ml cytosol	ER (fmoles/ml cytosol)	ER (fmoles/mg protein)
0	4.8	500	105
1.5	3.2	255	80
2	2.4	185	75
3	1.6	160	100
6	0.8	90	115
8	0.6	60	95
10	0.5	45	95

Human breast tumor cytosol (w/v-ratio, 1/4) was diluted with different volumes of buffer and at each dilution incubated with 6 concentrations of ^{3}H-E_2, allowing Scatchard analysis.

Table 2.4 Influence of dilution of human breast tumor cytosols on the measurement of ER binding sites.

Dilution factor	mg protein / ml cytosol	ER (fmoles/ml cytosol)	ER (fmoles/mg protein)
0[a]	6	55	9
1.3	4.5	35	8
2	3	0	0
0[b]	4.6	425	95
1.5	3.05	310	100
2	2.3	195	85
4	1.1	85	75
7	0.65	0	0

Undiluted (w/v-ratio, 1/4) and diluted cytosols were incubated with 6 concentrations of ^{3}H-estradiol, allowing Scatchard analysis.

[a]Cytosol A and [b]Cytosol B.

Table 2.2 shows that dilution of calf uterus cytosol, up to a w/v-ratio of 1/36 (0.3 mg protein/ml cytosol) had no systematic influence on the measured concentration of PgR binding sites (670 fmoles/mg protein). In addition, dilution of a human breast tumor cytosol did not have a marked influence on the amount of LR measured (Table 2.3). At a ten-fold dilution of the cytosol an ER value of 95 fmoles/mg protein (45 fmoles/ml cytosol) was measured, whereas in undiluted cytosol the value amounted to 105 fmoles/mg protein.

In contrast to the foregoing observations it appeared from the following experiments, that dilution certainly can have pronounced effects on the ER content measured.

A calf uterus cytosol with a w/v-ratio of 1/4, containing ER in an amount of 315 fmoles/mg protein (3.2 mg protein/ml cytosol) was diluted 10-, 20- and 30-fold. The linear Scatchard plots obtained (Fig. 2.4a,b,c), analysing the undiluted, 10- and 20-fold diluted cytosols, revealed a slight decrease of ER content from 315 to 290 and 250 fmoles/mg protein, respectively (K_d-values: 0.6, 1.3 and 1.6 nM). At the 30-fold dilution of the cytosol the Scatchard plot (Fig. 2.4d) did not reveal the presence of high affinity binding sites any more. At the 20-fold dilution, that is the highest dilution in which high affinity binding sites were still present, the difference between B_T and B_{NS} was 70 for the lowest ^3H-E_2 concentration (1.0 nM) and 190 counts/2 min/100 µl supernatant for the highest ^3H-E_2 concentration (8 0 nM). From this experiment it appeared that the lowest concentration of estradiol receptor binding sites, which still yielded an acceptable linear Scatchard plot - with a low K_d-value - was 2 fmoles/50 µl cytosol or 40 fmoles/ml cytosol. This value can be regarded as the lower limit of sensitivity. At this extreme cytosol dilution (w/v-ratio, 1/80) the protein concentration of the cytosol was 0.16 mg/ml and therefore the lower limit of sensitivity expressed as fmoles per mg protein was calculated to be 250.

The lowest estradiol receptor value - expressed as fmoles/mg protein - actually measured in a cytosol of a human breast tumor was 2.4 fmoles/50 µl cytosol (Fig. 2.5a) or 48 fmoles/ml cytosol. This value approached the above mentioned lower limit of sensitivity very well. In this tumor cytosol the protein content was 9.8 mg/ml and therefore the lower limit of sensitivity appeared to be 5 fmoles/mg protein. It appears that it has little sense to express the lower limit of sensitivity in fmoles/mg protein. Real information is given when the expression fmoles/ml cytosol is used.

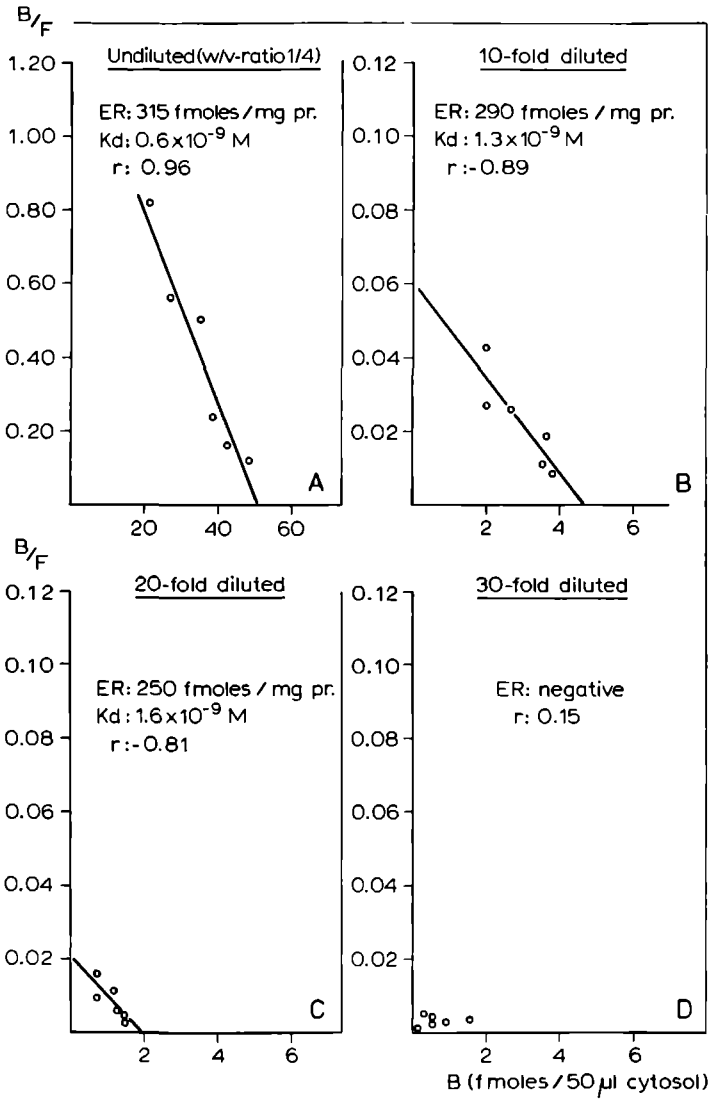


Fig. 2.4 A calf uterus cytosol with a w/v-ratio of 1/4 (3.2 mg protein/ml cytosol) was 10-, 20- and 30-fold diluted with buffer. The undiluted and diluted cytosols were incubated with 6 concentrations of ^{3}H-estradiol, allowing Scatchard analysis.

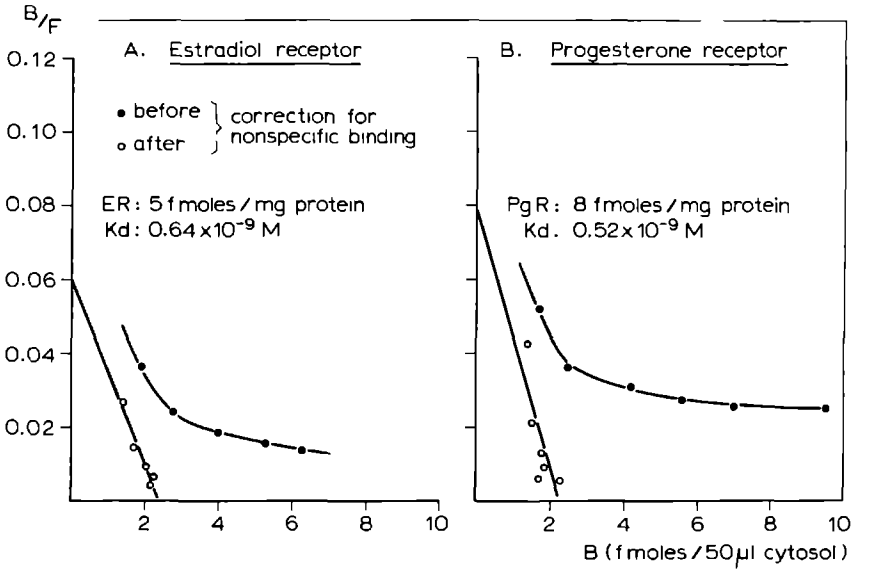


Fig. 2.5 Lowest concentration of estradiol and progesterone receptor binding sites expressed as fmoles/mg protein actually measured in cytosols of different human breast tumors. The protein content of the cytosols used for the determination of ER and PgR were 9.8 and 5.4 mg/ml cytosol respectively.

For the progesterone receptor the lowest value measured in another tumor cytosol was 46 fmoles/ml cytosol (Fig. 2.5b) or 8 fmoles/mg protein.

The effect of dilution of two different human breast tumor cytosols is shown in Table 2.4. In undiluted cytosol A an estradiol receptor concentration of 55 fmoles/ml cytosol (9 fmoles/mg protein) was measured. At a two-fold dilution of this cytosol no high affinity binding sites were measurable. In this cytosol dilution resulted in a value of ER under the lower limit of sensitivity. It is noted that the protein content of the two-fold diluted cytosol was 3.0 mg/ml. The undiluted (w/v-ratio, 1/4) tumor cytosol B showed an ER value of 425 fmoles/ml cytosol (95 fmoles/mg protein). A four-fold dilution resulted in a slight decrease of the ER-value (75 fmoles/mg protein). At seven-fold dilution the Scatchard plot did not allow interpretation (see Fig. 2.2F).

From calculation it followed that the ER content of this cytosol had to be 425/7 = 61 fmoles/ml cytosol and as such would remain detectable. Attention is drawn to the fact that the protein content of the cytosol was 0.65 mg/ml. It has been reported (Garola and McGuire, 1978; King et al., 1978) that ER measurements will be underestimated, when the protein content becomes lower than 1 mg/ml, an observation that was reconfirmed in the calf uterus cytosol dilution experiments described above (Table 2.1 and Fig. 2.4).

Low protein concentrations were often observed in cytosols, when small human breast tumor biopsies were analysed. Actually in 7% of 403 tumors assayed for ER, the protein concentration was smaller than 2 mg/ml cytosol. It is noteworthy that when the frequency of ER-positive tumors was plotted as a function of the protein concentration of the cytosol (Fig. 2.6), a sharp decline was observed at protein values lower than 2 mg/ml. Actually the frequency of ER-positive tumors declined from about 70% to 40% when the protein concentration fell in the range of 1-2 mg/ml and to 0% for the 5 tumor cytosols, which had a protein concentration below 1 mg/ml.

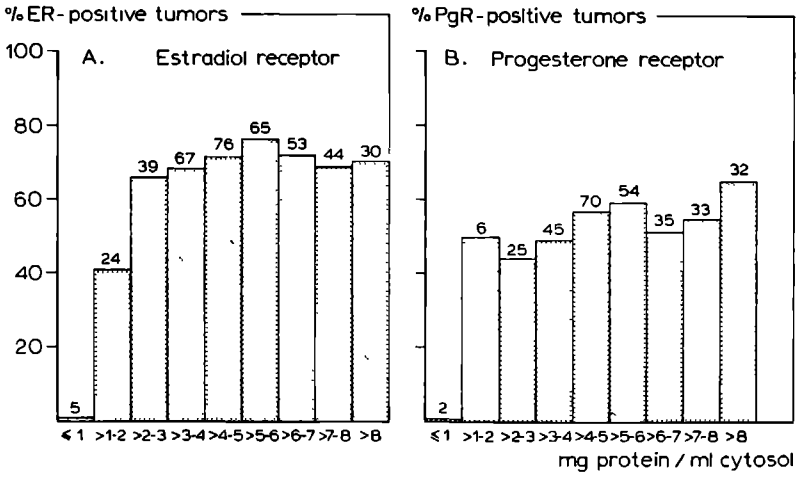


Fig. 2.6 The relation between the frequency of ER- and PgR-positive human breast tumors and the protein concentration of the analysed cytosols. The number of tumors assayed per group is indicated above the bars.

This decline of the frequency of ER-positive tumors very likely resulted from the fact that the lower limit of sensitivity was reached and in addition because ER-assays become unreliable at low protein concentrations.

Fig. 2.6b shows that the frequency of PgR-positive human breast tumors was not different for cytosols having a protein concentration above 2 mg/ml cytosol. Only 6 tumors were analysed with a protein content in the range of 1-2 mg/ml and 3 tumors were classified as PgR-positive. Both tumor cytosols, which revealed a protein concentration below 1 mg/ml appeared to be PgR-negative.

2.4. Reproducibility

The intra-assay variation of both the estradiol receptor and the progesterone receptor assay was estimated in 2 experiments with calf uterus tissue. The results, collected in Table 2.5 show coefficients of variation of 2.2 - 3.5 and 7.5 - 10.3% respectively. The inter-assay variation (Table 2.6) was analysed using both calf uterus and human breast tumor tissue. The coefficient of variation for the estradiol receptor assay was higher and amounted to 12.3 - 14.3%. The inter-assay variation coefficient for the progesterone receptor assay ranged between 8.1 - 12.3%. The inter-assay coefficient of variation appears to be not substantially different for these two target tissues containing receptor values ranging between 50 and 1000 fmoles/mg protein.

Table 2.5 Intra-assay variation of the estradiol and progesterone receptor determination.

	Estradiol Receptor		Progesterone Receptor	
number of determinations	5	7	5	7
mean ± SD (fmoles/mg protein)	91 ± 3	630 ± 14	210 ± 16	1100 ± 110
coefficient of variation (%)	3.5	2.2	7.5	10.3

Calf uterus tissue was vibrated to a fine powder. Parts of this powder were homogenised in cold buffer and separately analysed for ER and PgR binding sites.

Table 2.6 Inter-assay variation of the estradiol and progesterone receptor determination.

	Estradiol Receptor		Progesterone Receptor	
Calf uterus tissue:				
number of determinations	11	21	13	11
mean ± SD (fmoles/mg protein)	330 ± 40	630 ± 80	810 ± 87	1000 ± 120
coefficient of variation (%)	12.3	12.6	10.7	12.3
Human breast tumor tissue:				
number of determinations	7	9	6	
mean ± SD (fmoles/mg protein)	49 ± 7	225 ± 31	260 ± 21	
coefficient of variation (%)	14.3	13.8	8.1	

Tissue was pulverized in the frozen state and lyophilised in glass vials. The vials were stoppered whilst under vacuum and stored at 0° - 4°C. On various days lyophilised tissue was analysed for ER and PgR binding sites (see 2.7.3).

2.5.1 The steroid specificity of estradiol receptor binding sites

The steroid specificity of estradiol receptor binding was established by competition analysis, using $^{3}H\text{-}E_2$ as the radioactive ligand, and by direct binding studies using several radioactive estrogenic compounds. Competition analysis was performed with one concentration of $^{3}H\text{-}E_2$ (5 nM) and increasing concentrations of various nonradioactive compounds (4.10^{-9} - 4.10^{-5}M). It may be noted that all experiments were performed at 0 - 4°C and that the incubation time was 18 - 20 hours. An example of the competition of several estrogens or antiestrogens for $^{3}H\text{-}E_2$ binding to calf uterus is shown in Fig. 2.7. The results were expressed as relative binding affinities, according to the method described by Korenman et al. (1970). The relative binding affinity is defined as the relative concentrations of competitor and radioinert ligand required to displace 50% of bound ^{3}H-ligand from specific receptor binding sites. The results of a competition analysis, in which the steroid specificity of estradiol receptor present in three different target tissues was investigated, are collected in Table 2.7. The relative binding affinity for the binding to estradiol receptor sites present in calf uterus tissue and in DMBA-induced rat mammary tumor tissue was almost equal. The relative binding affinity for the binding sites present in biopsies of human breast tumors showed the same pattern, although in this particular experiment moxestrol and diethylstilbestrol were relatively strong competitors. The observation that progestins (R5020), androgens (DHT) and glucocorticoids (dexamethasone and cortisol) did not compete, even when present at 1000-fold molar excess, indicates that the binding to this receptor is highly estrogen specific. In addition the potent synthetic estrogenic compounds moxestrol and diethylstilbestrol displaced $^{3}H\text{-}E_2$ most effectively, whereas estriol and 17 α-estradiol were less active (Fig. 2.7). The antiestrogenic compounds nafoxidine and clomiphene competed only when present at high concentrations (Fig. 2.7).

It has to be emphasized that the relative binding affinity estimated from competition experiments depends on:

1. the incubation time and temperature (Bouton and Raynaud, 1978; Rochefort and Capony, 1977)
2. metabolism during the incubation

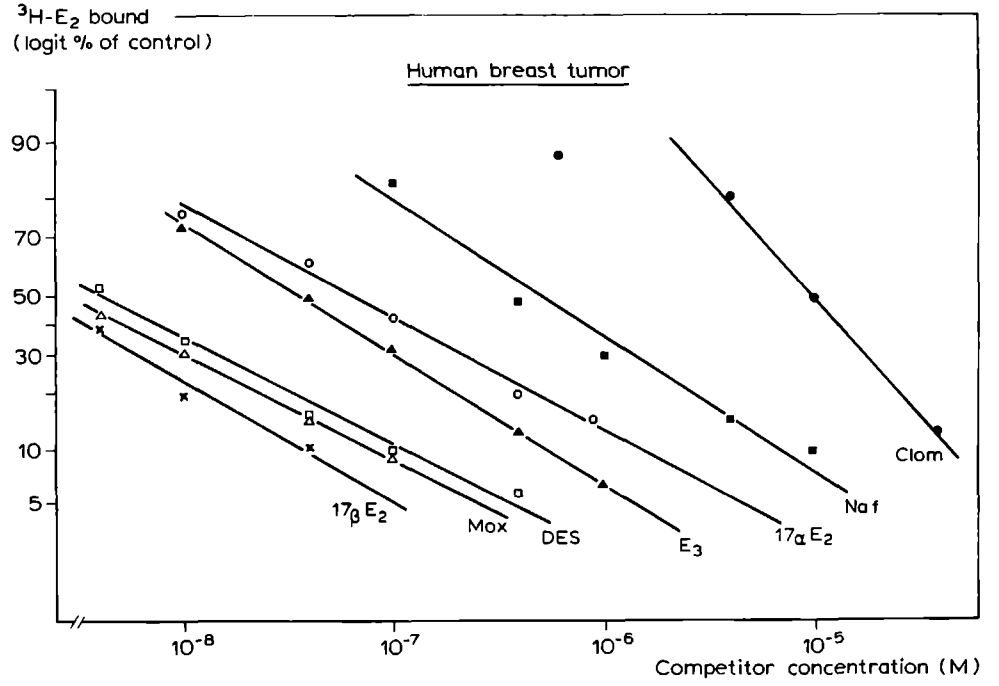


Fig. 2.7 Competition for ^{3}H-E$_2$ binding in human breast tumor cytosol. Tumor cytosol was incubated for 18 hr at 0 - 4°C with ^{3}H-E$_2$ (6 nM) in the absence or presence of increasing concentrations of 17 β-estradiol (17 β-E$_2$), moxestrol (Mox), diethylstilbestrol (DES), estriol (E$_3$), 17 α-estradiol (17 α-E$_2$), nafoxidine (Naf) and clomiphene (clom), ranging between 4 x 10^{-9} and 4 x 10^{-5}M.

3. the true competitor concentration (adsorption, solubility) (Capony and Rochefort, 1978; Nicholson et al., 1977)
4. the presence of nonspecific binding proteins (Capony and Rochefort, 1978; Nicholson et al., 1977).

Table 2.7 Competition for ^{3}H-Estradiol binding in cytosol from calf uterus tissue, DMBA-induced mammary tumors and from human breast tumors.

Competitor	Calf uterus	DMBA-mammary tumor	human breast tumor
	Relative Binding Affinity (%)[a]		
Estradiol	100	100	100
Moxestrol	70	55	90
Diethylstilbestrol	50	40	80
Estriol	6	8	5
17 α-Estradiol	3	4	2
Nafoxidine	0.4	0.3	0.2
Clomiphene	0.02	0.04	0.02
R5020	<0.01	<0.01	<0.01
5 α-Dihydrotestosterone	<0.01	<0.01	<0.01
Dexamethasone	<0.01	<0.01	<0.01
Cortisol	<0.01	<0.01	<0.01

Cytosol was incubated with 5.10^{-9}M ^{3}H-Estradiol in the presence of competing steroids (4.10^{-9} to 4.10^{-5}M).

[a]Relative binding affinity is defined as the relative concentrations of competitor and radioinert ligand required to displace 50% of bound radioligand from receptor binding sites.

Because of these influences the K_d-values of five estrogenic compounds were determined by competitive experiments using ^3H-E_2 and also by direct binding studies using the radioactive forms of these compounds. The K_d-values of these experiments are collected in Table 2.8. ^3H-estradiol, ^3H-ethynylestradiol, ^3H-estriol, ^3H-moxestrol and ^3H-estrone all showed linear Scatchard plots, after correction for nonspecific binding, revealing about the same concentration of specific binding sites (data not shown). From these direct binding studies it appeared that estradiol and ethynylestradiol have equal K_d-values. The K_d-values of estriol, moxestrol and estrone were 3.5, 5 and 6 times higher. Competitive experiments revealed K_d-values, which were equal to those obtained with direct binding studies only for estradiol. The K_d-values obtained for ethynylestradiol, estriol and estrone, using competitive experiments with ^3H-E_2, were 2 to 8.5-fold higher than those obtained in direct binding studies. The K_d-value obtained for moxestrol however, using competitive experiments, was 4-times lower than the average K_d-value obtained in the direct binding studies. Interestingly the direct binding studies revealed that the nonspecific binding of ^3H-ethynylestradiol was 3-fold higher and the nonspecific binding of ^3H-moxestrol was generally lower than that of ^3H-E_2 (2.5.2.5). From these observations it appeared that in competitive experiments with ^3H-E_2 in the case of moxestrol the K_d may be underestimated and thereby the affinity overscored, when compounds have a lower nonspecific binding than estradiol. On the other hand the K_d in competitive studies may be overestimated, when compounds have a higher nonspecific binding than estradiol (e.g. ethynylestradiol).

2.5.2 Interference of plasma proteins with the assay of estradiol receptor binding sites

Cytosol prepared from human breast tumor biopsies may be contaminated with plasma proteins. Plasma contains proteins which nonspecifically (albumin) or specifically (Sex Hormone Binding Globulin, SHBG) bind estradiol. SHBG binding sites, in contrast to albumin, can be saturated by 10^{-6}M estradiol. It has been reported that the albumin content of human breast tumor cytosols ranged from 3-52% of the total protein concentration (Maass et al., 1975; Teulings et al., 1975; Wagner and Jungblut, 1976; Netten et al., 1977), whereas according to one study (Wagner and Jungblut, 1976) the SHBG concentration ranged between 100-710 fmoles/mg total protein.

Table 2.8 Comparison of the equilibrium dissociation constant (K_d) of estrogenic compounds for calf uterus receptor sites determined by direct binding studies or by competitive experiments with $^3H\text{-}E_2$.

Compound	Direct binding Studies[a] K_d: 10^{-9}M	Competition studies[b] K_d: 10^{-9}M
Estradiol	0.18 ± 0.18 (7)	0.15
Ethynylestradiol	0.14 (2)	1.2
Estriol	0.61 (2)	1.5
Moxestrol	0.90 ± 0.49 (7)	0.20
Estrone	1.05 (2)	2.2

[a] Calf uterus cytosol was incubated with six different concentrations of ^3H-ligand (1-8 nM) in the absence or presence of 10^{-6}M radioinert ligand. The K_d-values were calculated from the slope of the Scatchard plot. The number of different determinations is indicated in parentheses.

[b] Increasing concentrations of $^3H\text{-}E_2$ (1-8 nM) were incubated in the absence or presence of two concentrations of nonradioactive competitor (10^{-8} and 10^{-7}M). The K_d-values were calculated from the slopes of double reciprocal plots, according to the following equation: slope $^3H\text{-}E_2$ + competitor / slope $^3H\text{-}E_2$ = 1 + competitor/K_d competitor. The indicated K_d-value for each compound is the mean of the two values calculated for the two competitor concentrations.

Therefore it was investigated whether plasma contamination seriously interferes with the estradiol receptor assay as described under 2.2.3.

2.5.2.1 Interference of albumin contamination

The interference of albumin with the assay of estradiol receptors was mimicked by the addition of albumin to calf uterus cytosol. The results of one of these experiments are shown in Table 2.9. In this experiment the albumin content ranged between 0 and 48% of the total protein content. The addition of increasing concentrations of albumin had no influence on the measurement of ER-binding sites in calf uterus cytosol. The ER-values ranged from 80-85 fmoles/mg calf uterus cytoplasmic protein. The measured K_d-values however increased with the addition of albumin.

2.5.2.2 Interference of serum contamination

Diluted human sera from two premenopausal women were added to calf uterus cytosol (Table 2.10). The concentration of ER binding sites of this cytosol was 185 fmoles/mg protein. The serum content of the prepared cytosols varied between 67 and 80%. As is indicated by the correlation coefficients ($r = 0.99$) perfect linear Scatchard plots were obtained after correction for nonspecific binding with 10^{-6}M radioinert estradiol. Table 2.10 shows that even these high serum contaminations had no influence on the estimation of ER binding sites. The K_d-values however again increased considerably by the addition of serum proteins.

Control studies of $^3H\text{-}E_2$ binding to these diluted sera revealed, that in this range of $^3H\text{-}E_2$ concentrations (1-8 nM) the B/F ratio appeared to be constant ($B/F_T = 0.04$) and 70% of this binding appeared to be nonspecific ($B/F_{NS} = 0.028$, results not shown).

The SHBG level in plasma from pregnant women in the third trimester is about 3 to 4 times higher than in plasma of normal premenopausal women (Anderson et al., 1972). Therefore third trimester human pregnancy plasma was used to evaluate whether extensive SHBG contamination interferes with the estradiol receptor assay.

Table 2.9 Influence of the addition of albumin on the determination of estradiol receptor binding sites in calf uterus cytosol.

Calf uterus cytosolic protein (mg/ml)	Albumin (mg/ml)	ER (fmoles/ml cytosol)	K_d (nM)
6.5	-	545	0.05
6.5	2	530	0.08
6.5	3	525	0.10
6.5	4	540	0.11
6.5	5	545	0.11
6.5	6	545	0.12

Calf uterus cytosol was analysed for ER, after the addition of increasing concentrations of BSA.

Table 2.10 Influence of human serum proteins on the determination of estradiol receptor binding sites in calf uterus cytosol.

Calf uterus protein (mg/ml cytosol)	Serum protein[a] (mg/ml cytosol)	r[b]	ER (fmoles/ml cytosol)	K_d (nM)
5.7	-	0.982	1055	0.43
4.3	8.6[c]	0.996	1085	1.02
3.2	13.2[c]	0.995	1020	1.14
4.3	8.9[d]	0.996	1090	1.08
3.2	12.8[d]	0.995	1080	1.45

8-fold diluted human serum of two premenopausal women was added to calf uterus cytosol. The resulting cytosols were analysed for ER-activity as described under 2.2.3.

[a]As a control 8-fold diluted human sera were incubated with ^{3}H-E_2 (1-8 nM) in the absence (B_T) or presence (B_{NS}) of 10^{-6}M radioinert estradiol.

[b]Least squares correlation coefficient

[c]Serum of a premenopausal woman

[d]Serum of a premenopausal woman using oral contraceptives.

Five-fold diluted third trimester human pregnancy plasma was added to calf uterus cytosol and incubated with increasing concentration of $^{3}H\text{-}E_2$ (Fig. 2.8). In this particular experiment the nonspecific binding was determined in the presence of 5×10^{-5}M clomiphene. This antiestrogen, in contrast to estradiol, does not bind specifically to plasma proteins. The plasma protein content in this experiment was 74% of the total protein concentration. Saturation of specific estradiol receptor binding sites present in calf uterus cytosol was attained at approximately 2 nM (Fig. 2.8).

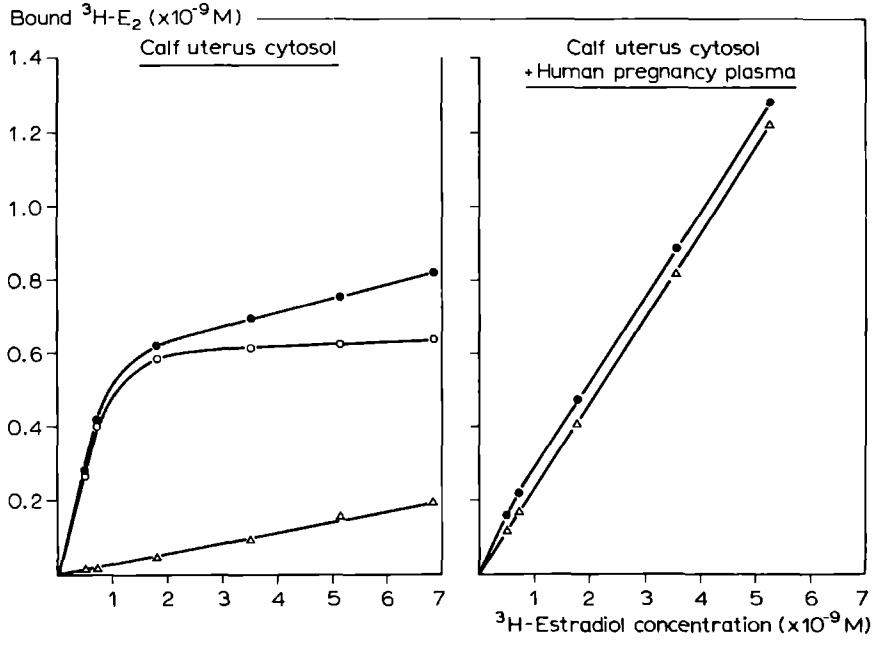


Fig. 2.8 Influence of plasma proteins on the measurement of estradiol receptor binding sites. Calf uterus cytosol and calf uterus cytosol plus five-fold diluted third trimester human pregnancy plasma was incubated with increasing concentrations of $^{3}H\text{-}E_2$ in the presence of 5×10^{-5}M clomiphene. After 18 hr at 0 - 4°C, the amounts of total binding (●), nonspecific binding (Δ) and specific binding (O) were determined by the DCC-assay.

Scatchard analysis of these data gave a linear plot with a K_d of 0.17 nM and a binding capacity of 325 fmoles/mg protein. No saturation of binding sites was observed for the plasma contaminated cytosol. Clomiphene showed only a minor competition for this ^3H-E_2 binding, indicating that almost all radioactive ligand is bound to plasma proteins. Thus under conditions of extreme contamination of the cytosol with SHBG, binding of ^3H-E_2 to estradiol receptor binding sites may become undetectable.

For comparison the binding of ^3H-E_2 to pregnancy plasma was studied. Diluted pregnancy plasma (16 mg protein/ml) was incubated with estradiol concentrations, ranging between 1.0 and 10.000 nM (Fig. 2.9). The binding ultimately was clearly saturable and again the B/F-ratio appeared to be constant in the concentration range from 1-8 nM. A decline of the B/F-ratio occurred, when the estradiol concentration amounted to 85 nM. After correction for nonspecific binding according to the method by Rosenthal (1967) a linear Scatchard plot (r = 0.98) was obtained with a K_d-value of 60.10^{-9}M. This K_d-value was at least 15 times higher than the K_d-values observed in tumor cytosols (2.3.1).

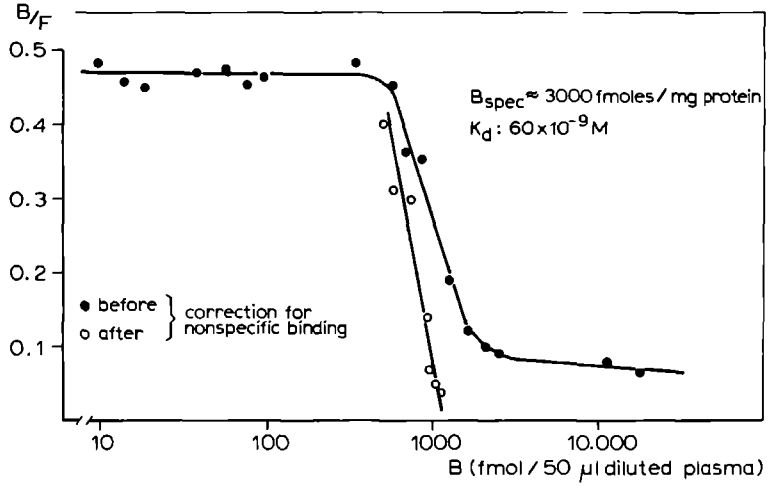


Fig. 2.9 Five-fold diluted third trimester human pregnancy plasma was incubated with increasing estradiol concentrations ranging between 1.0 and 10.000 nM. The Scatchard plot of total binding (●) was corrected for nonspecific binding, according to Rosenthal (1967), to obtain the Scatchard plot of specific binding (○).

2.5.2.3 Stripping of estradiol bound to low affinity binding sites by dextran-coated charcoal

The effect of stripping of $^{3}H\text{-}E_2$ bound to proteins of diluted human pregnancy plasma and to proteins of breast tumor cytosol during continuously shaking with dextran-coated charcoal is shown in Fig. 2.10. Under the conditions chosen estradiol bound to plasma proteins dissociates very rapidly ($t_{\frac{1}{2}}$ = 10 - 15 min), whereas the dissociation of estradiol from its receptor appears to be very slow. Furthermore it was observed that within the normally used contact time of 10 min all estradiol previously bound dissociates from albumin (results not shown). Thus the binding of $^{3}H\text{-}E_2$ to SHBG and to albumin is completely eliminated when a prolonged charcoal contact time of 90 min is used. Therefore the estimation of estradiol receptors with the normally used contact time of 10 min was compared with a contact time of 90 min. The results of these experiments, in which cytosols of calf uterus tissue and of human breast tumor biopsies were analysed, are collected in Fig. 2.11.

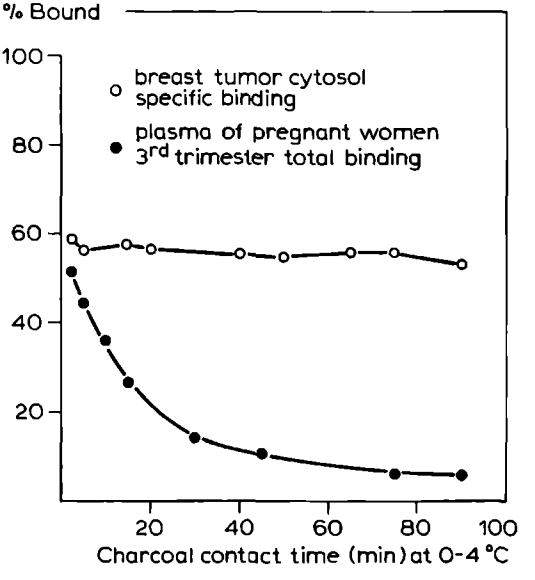


Fig. 2.10 Cytosol of a human breast tumor (○) and 5-fold diluted third trimester human pregnancy plasma (●) were incubated with $^{3}H\text{-}E_2$ (5 nM) for 18 hr at 0 - 4°C. After the addition of dextran-coated charcoal the microtiter plates were continuously shaken for the indicated times on a mechanical shaker.

The receptor values obtained after 90 min contact time generally were lower than the corresponding values obtained after 10 min of charcoal treatment. However, with the exception of one tumor the differences were negligible.

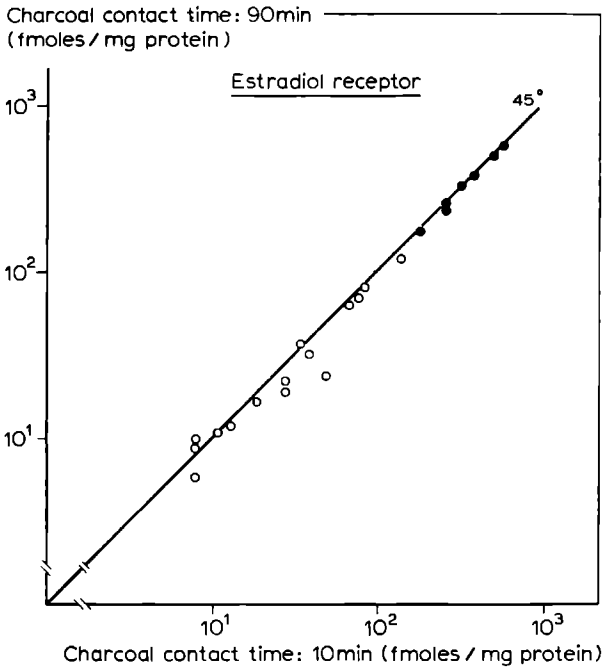


Fig. 2.11 Influence of the dextran-coated charcoal contact time on the estimation of ER binding sites in calf uterus tissue (n=7, ●) and in human breast tumor tissue (n=22, ○). Cytosol was incubated in two microtiter plates with 6 concentrations of ^3H-E_2, allowing Scatchard analysis. Free and weakly bound steroid was removed by adding dextran-coated charcoal. One of the microtiter plates was shaken for 10 and the other for 90 min. Seven tumor cytosols were regarded as estradiol receptor-negative with both procedures.

2.5.2.4 Nonspecific binding determined with an excess of estradiol, diethylstilbestrol or moxestrol.

Estrogens such as diethylstilbestrol (DES) of moxestrol (Roussel Uclafs compound R2858) show high affinity for estradiol receptor binding sites (2.5.2). These compounds, in contrast to estradiol, do not bind to specific plasma proteins (Raynaud et al., 1978; Wittliff, 1975). Therefore the difference in saturable binding using a large excess of radioinert estradiol compared to a large excess of DES (or moxestrol) represents estradiol binding to SHBG. To detect any SHBG interference the nonspecific binding in 49 human breast tumor cytosols was determined with 10^{-6} M DES and 10^{-6}M E_2 and in 27 tumor biopsies with 10^{-6}M moxestrol and 10^{-6}M E_2, using ^{3}H-E_2 as the radioactive ligand. The results of these experiments are collected in Fig. 2.12 and 2.13. All tumors classified as estradiol receptor-negative using an excess of radioinert E_2 were also regarded as receptor-negative using DES (n=25) or moxestrol (n=12). For the tumors regarded as receptor-positive, it was observed that the quantitative receptor values obtained after correction for nonspecific binding with E_2, were equal to or slightly exceeded the values using an excess of DES or moxestrol. This overestimation of ER binding sites using radioinert E_2 occurred only in tumors showing relatively low receptor concentrations. In these series of tumors the values obtained with radioinert E_2 never exceeded the receptor value obtained with DES or moxestrol more than 40%. Two tumors revealed, after correction for nonspecific binding with E_2, the type of nonlinear Scatchard plot illustrated in Fig. 2.2B. However the Scatchard plots of these tumors were linear after correction for nonspecific binding with DES and moxestrol respectively indicating that in these two tumors SHBG probably interfered with the assay of ER binding sites.

2.5.2.5 Estimation of estradiol receptor binding sites assayed with estradiol and moxestrol as the radioactive ligands

As was mentioned above and recently emphasized by Raynaud et al. (1978) ^{3}H-moxestrol does not bind to SHBG. Under the conditions of our receptor assay method it appeared that this compound only showed a very low nonspecific binding even to human pregnancy plasma (B/F_{NS} = 0.02). Therefore ^{3}H-moxestrol is particularly useful in the assay of ER binding sites present in human breast tumor cytosol, which may be contaminated with plasma proteins.

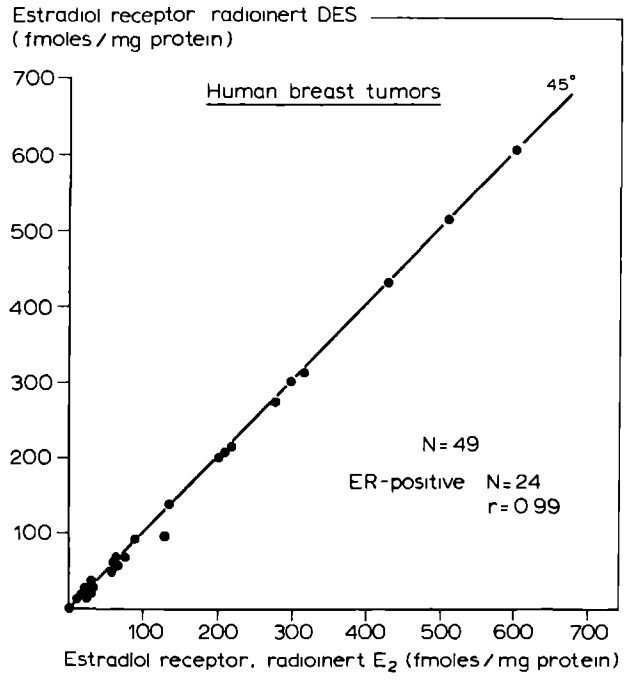


Fig. 2.12
Correlation between the concentration of specific estradiol receptor binding sites in 49 human breast tumor cytosols after elimination of the nonspecific binding with 10^{-6}M radioinert E_2 and with 10^{-6}M radioinert diethylstilbestrol.

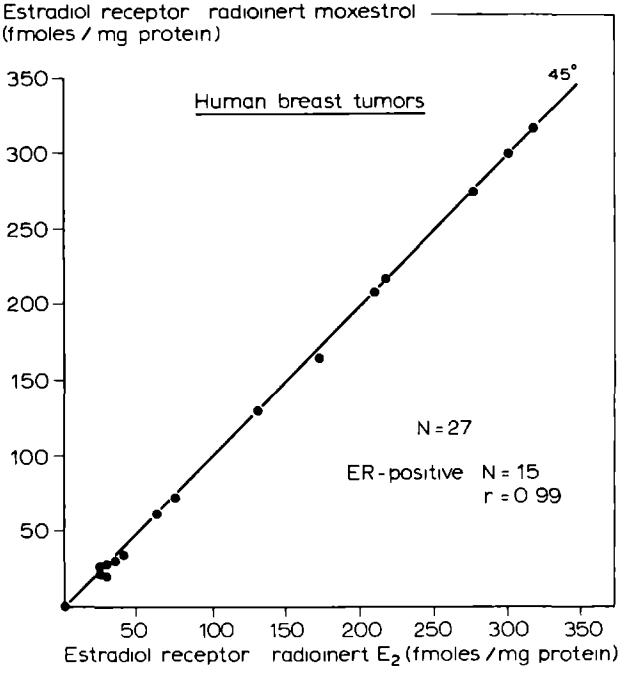


Fig. 2.13
Correlation between the concentration of specific estradiol receptor binding sites in 27 human breast tumor cytosols after elimination of the nonspecific binding with 10^{-6}M radioinert E_2 and with 10^{-6}M radioinert moxestrol.

The correlation between ER binding sites assayed with ^{3}H-moxestrol and with $^{3}H\text{-}E_2$ in 65 human breast tumor biopsies can be seen in Fig. 2.14. Twenty tumors (39%) were regarded as receptor-negative and 39 tumors (60%) as receptor-positive using either radioactive ligand. The quantitative receptor values of these 39 tumors agreed very well (r = 0.96). The dissociation constant for the moxestrol: receptor complex was 1.4 $\pm$ 1.4 nM.

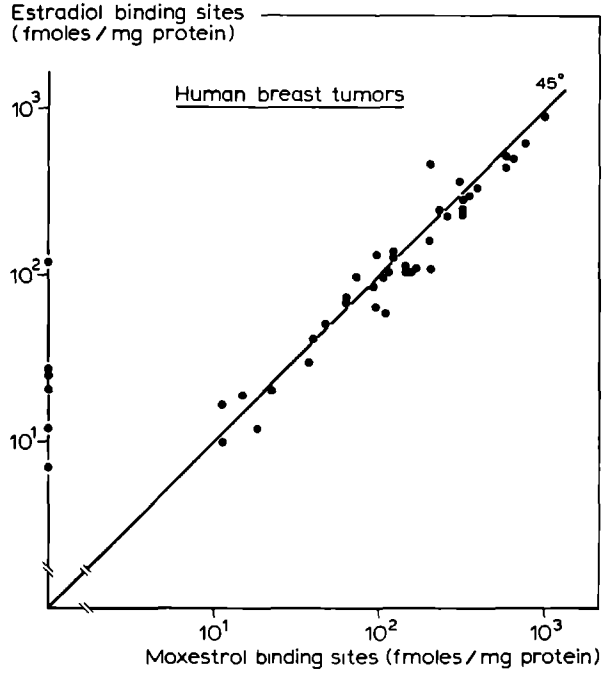


Fig. 2.14 Correlation between the concentration of estradiol receptor binding sites assayed with $^{3}H\text{-}E_2$ (1-8 nM) and ^{3}H-moxestrol (1-8 nM) in 65 human breast tumor cytosols. The nonspecific binding was determined in the presence of 10^{-6}M nonradioactive E_2 and moxestrol respectively. Twenty tumors were regarded as receptor-negative using both radioactive ligands.

The corresponding value for the estradiol: receptor complex in the same breast tumor cytosols was 0.40 ± 0.30 nM. Furthermore it was observed that the non-specific binding was generally 2 times lower with ^{3}H-moxestrol than with ^{3}H-estradiol. Attention is drawn to the fact that six tumors (9%) had to be classified as receptor-positive using ^{3}H-E_2 and as receptor-negative using moxestrol as the radioactive ligand. Although five of these tumors had low receptor values (<30 fmoles/mg protein), the observed K_d-values of these tumors ranged between 0.22 and 3.5 nM, indicating that in these tumors estradiol was specifically bound to the receptor and not exclusively to SHBG. The observation that 4 of these 6 tumors revealed detectable amounts of PgR furthermore indicates that estradiol receptors are present in these tumors, because the synthesis of progesterone receptor is induced by estradiol (Wiest and Rao, 1971). Interestingly no tumors classified as receptor-positive using ^{3}H-moxestrol were estimated as receptor-negative using ^{3}H-estradiol.

2.6 Specificity of the progesterone receptor assay

2.6.1 The synthetic progestins R5020 and Org 2058 as radioactive ligands for the progesterone receptor assay

The assay of the cytoplasmic progesterone receptor has been seriously hampered by the fact that progesterone - besides to the receptor protein - binds also tightly to certain plasma proteins as the corticosteroid binding globulin and to the glucocorticoid receptor. Furthermore progesterone dissociates very rapidly from its receptor binding sites (Milgrom et al., 1972; Feil et al., 1972). It has been reported that a new synthetic progestin, ^{3}H-R5020 (Philibert and Raynaud, 1973) shows high affinity for progesterone receptor binding sites in normal progesterone target tissues such as uterus, pituitary, hypothalamus and in mammary tumor tissue (Horwitz and McGuire, 1975; Raynaud, 1977). This compound does hardly display affinity towards CBG and has the additional advantage that its complex with the progesterone receptor displays a much lower tendency to dissociate. For these reasons ^{3}H-R5020 was used as the radioactive ligand in the assay of PgR. In this evaluation another synthetic progestin ^{3}H-Org 2058 has been used, which also binds to the progesterone receptor with high affinity under formation of a slowly dissociating complex (Jänne et al., 1976).

The progesterone receptor sites of 72 human breast tumor specimens were estimated with R5020 and Org 2058 as the radioactive ligands. The results are presented in Fig. 2.15. Thirty tumors were regarded as receptor-negative with both radioactive ligands. The linear Scatchard plots of 41 tumors revealed high affinity binding sites (K_d<4 nM) and were therefore classified as receptor-positive. As can be derived from Fig. 2.15 no systematic difference between the quantitative receptor values was observed (r = 0.98). Only one tumor was classified as receptor-positive using ^{3}H-R5020 and as receptor-negative using ^{3}H-Org 2058. The K_d-values obtained with ^{3}H-R5020 and ^{3}H-Org 2058 were about the same: 0.90 $\pm$ 0.80 and 0.80 $\pm$ 0.70 nM respectively.

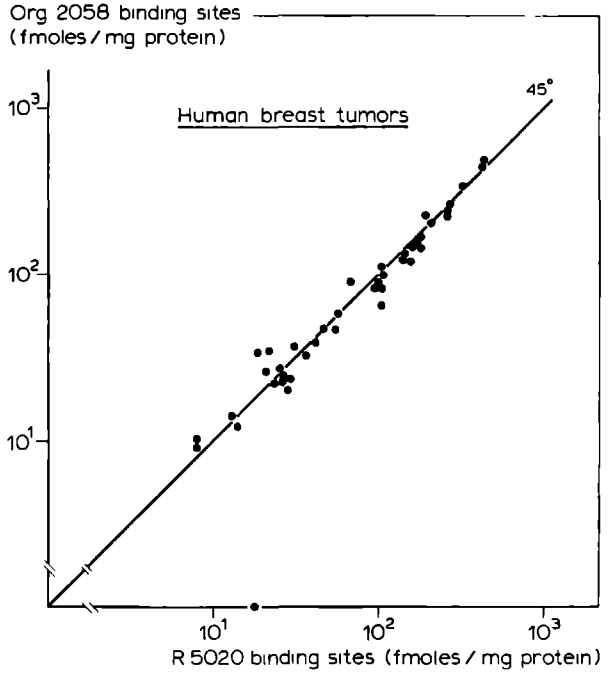


Fig. 2.15 Correlation between the concentration of progesterone receptor binding sites assayed with 1-10 nM ^{3}H-R5020 and ^{3}H-Org 2058 in 72 human breast tumor cytosols. The nonspecific binding was determined in the presence of 10^{-6} nonradioactive R5020 and Org 2058 respectively. Thirty tumors were regarded as receptor-negative using both radioactive ligands.

The same type of study was performed using 36 DMBA-induced rat mammary tumor biopsies. Eleven tumors were regarded as receptor-negative and 25 tumors as receptor-positive using both radioactive ligands. Again the quantitative receptor values obtained with ^{3}H-R5020 and ^{3}H-Org 2058 agreed very well ($r = 0.98$).

2.6.2 The steroid specificity of progesterone receptor binding sites

The steroid specificity of PgR binding sites was studied with both radioactive ligands. Fig. 2.16 shows the displacement of ^{3}H-Org 2058 bound to human breast tumor cytosol with several nonradioactive competitors. It can be seen that Org 2058 and R5020 were approximately 10- to 20-fold more potent than the natural hormone progesterone. The anti-androgen cyproterone acetate and the synthetic glucocorticoid triamcinolone acitonide, both of which possess progestational activity, have a low displacing capacity. Estradiol and 5 α-dihydrotestosterone competed only when present in excessive amounts. Interestingly the glucocorticoids cortisol and dexamethasone showed no competition when present at 1000-fold molar excess. Identical results were obtained when cytosols of human breast tumors were analysed with ^{3}H-Org 2058 or ^{3}H-R5020 (Table 2.11). This makes it highly probable that both ligands bind to the same receptor binding sites.

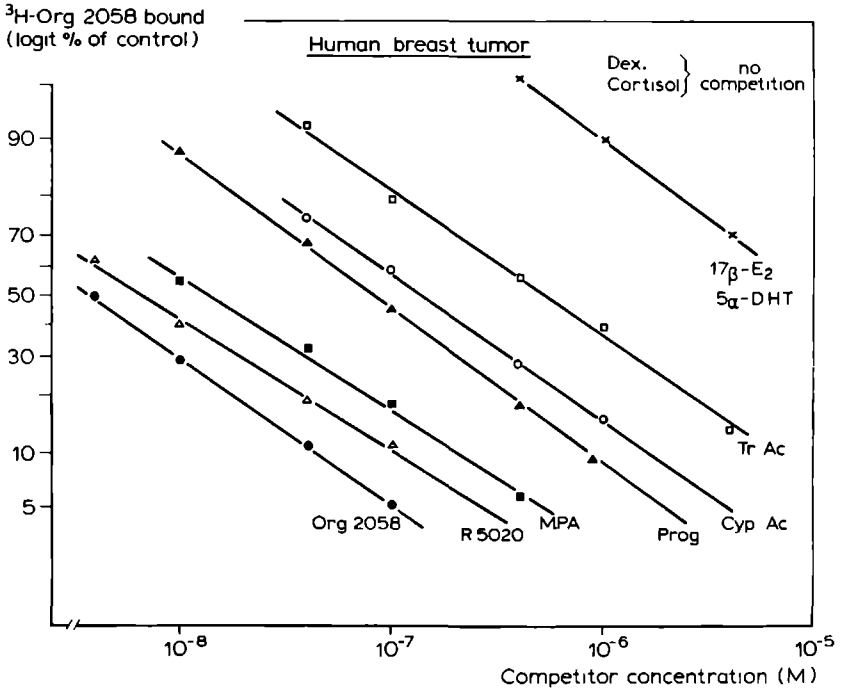


Fig. 2.16 Competition for ^{3}H-Org 2058 binding in cytosol from a human breast tumor. Tumor cytosol was incubated for 18 hr at 0 - 4°C with ^{3}H-Org 2058 (7 nM) in the absence or presence of increasing concentrations of Org 2058, R5020, medroxyprogesterone acetate (M.P.A.), progesterone (Prog.), cyproterone acetate (Cyp. Ac.), triamcinole acetonide (Tr. Ac.), 17 β-estradiol (17 β-E_2), 5 α-dihydrotestosterone (5 α-D.H.T.), dexamethasone (Dex.) and cortisol, ranging between 4 x 10^{-9} and 4 x 10^{-6}M.

Table 2.11 Competition for ^{3}H-Org 2058 and ^{3}H-R5020 binding in cytosols from human breast tumors.

Competitor	^{3}H-Org 2058	^{3}H-R5020
Org 2058	100	100
R5020	60	50
Medroxyprogesterone acetate	30	30
Progesterone	5	5
Cyproterone acetate	3	3
Triamcinolone acetonide	0.7	0.8
Estradiol	0.04	0.04
5 α-dihydrotestosterone	0.04	0.04
Cortisol	<0.01	<0.01
Dexamethasone	<0.01	<0.01

Human breast tumor cytosol was incubated with 8 nM ^{3}H-Org 2058 and 8 nM ^{3}H-R5020 in the absence or presence of increasing concentrations (4.10^{9} to 4.10^{-6}M) of various nonradioactive competitors. The results were expressed as the relative binding affinity, which is defined as the relative concentration of competitor and radioinert ligand required to displace 50% of bound radioligand from its receptor binding sites. The relative binding affinity of Org 2058 was taken to be 100.

2.6.3 Interference of glucocorticoid receptor proteins

It has been demonstrated that R5020 besides to the progesterone receptor binds also to the glucocorticoid receptor, albeit with low affinity (Raynaud, 1977). Lippman et al. (1977) estimated a K_d-value of 20 nM for the binding of R5020 to glucocorticoid receptors and emphasized, that the use of R5020 could lead to an overestimation of the concentration of high affinity PgR binding sites, when glucocorticoid receptors are present. Therefore it was investigated whether the presence of glucocorticoid receptors interferes with our progesterone receptor assay.

Rat liver, which contains high concentrations of glucocorticoid receptors, was used to study the binding properties of R5020 and Org 2058 to these receptors. Rat liver cytosol was incubated with ^{3}H-dexamethasone and increasing concentrations of radioinert R5020 or Org 2058 (10-40,000 nM) and dexamethasone. These displacement studies revealed that R5020 as a competitor was about 30 times less potent (Relative Binding Affinity : 3.5) than dexamethasone. Org 2058 however competed only weakly for these receptor binding sites (Relative Binding Affinity : 0.12). Direct binding studies of ^{3}H-dexamethasone, ^{3}H-R5020 and ^{3}H-Org 2058 to rat liver cytosol showed that only dexamethasone was bound with high affinity. After correction for nonspecific binding a straight Scatchard plot was obtained with a K_d of 3.5×10^{-9}M and the concentration of specific dexamethasone binding sites was 375 fmoles/mg protein. ^{3}H-R5020 and ^{3}H-Org 2058 however only demonstrated nonsaturable binding (B_{FNS}= 0.05). The direct interference of glucocorticoid receptors with the determination of progesterone receptors was mimicked by addition of rat liver cytosol to calf uterus cytosol. In these studies progesterone receptor sites were determined with ^{3}H-R5020, because the displacement studies indicated that interference can only be expected, when the receptor assay is performed with this radioactive ligand. The results of these experiments are summarized in Table 2.12. After correction for nonspecific binding, linear Scatchard plots were obtained (r = 0.98-0.99, Table 2.12) suggesting that R5020 was bound to a single class of binding sites. The addition of liver cytosol caused no or only a slight increase in the concentration of receptor binding sites, whereas the K_d-value remained the same.

Table 2.12 Analysis of interference of glucocorticoid receptors with the assay of progesterone receptor binding sites using R5020 as the radioactive ligand.

Calf uterus cytosol (mg protein/ml incubate)	Rat liver cytosol[a]	r[b]	K_d (10^{-9}M)	Receptor sites[c]
5.2	-	0.99	1.6	545
5.2	1.2	0.98	1.4	605
5.2	2.4	0.99	1.5	580
5.2	4.8	0.99	1.6	560

Different dilutions of rat liver cytosol were added to a constant volume of calf uterus cytosol. Progesterone receptor binding sites were determined with ^{3}H-R5020 as previously described (2.2.3).

[a]Rat liver cytosol was incubated with ^{3}H-Dexamethasone in the absence or presence of 10^{-6}M radioinert dexamethasone. Scatchard analysis revealed a K_d of 1.0 nM and the concentration of receptor sites was 305 fmoles/mg protein.

[b]Least squares correlation coefficient.

[c]fmoles/mg calf uterus cytoplasmic protein.

2.6.4 Stripping of radioactive ligand by dextran-coated charcoal

The determination of receptor binding sites as performed in the present study, using charcoal for separation of free and bound steroid, occurs under non-equilibrium conditions. This implies that only tritiated ligands may be used, which dissociate slowly from the receptor binding sites. The dissociation of bound ^{3}H-R5020 and ^{3}H-Org 2058 to human breast tumor cytosol during continuously shaking with DCC (0 - 90 min) showed bifasic curves. A steep fall was observed during the first 10 min, probably representing stripping of low affinity binding.

Between 20 and 90 min the decrease in radioactivity became relatively slow, probably due to dissociation of these progestins from the specific receptor binding sites. The dissociation rate constants (20 - 90 min) were calculated to be 0.61×10^{-4} and 0.69×10^{-4} s^{-1} for R5020 and Org 2058 respectively. From these rate constants it follows that during the normally used contact time of 10 min the dissociation of specifically bound ^{3}H-ligand is less than 5%.

2.7 The effect of storage of target tissue on cytoplasmic receptor binding sites

2.7.1 Storage of DMBA-induced rat mammary tumor tissue at -70°C

DMBA-induced mammary tumor tissues were analysed for ER- and PgR-binding sites before and after storage for 15 months in a Revco Ultra Deepfreezer (-70°C). Before storage the ER levels ranged between 25 and 225 fmoles/mg protein, and after storage the ER levels in all tumors (n=11) had decreased and now ranged between 15 and 85 fmoles/mg protein. The average decrease was 50%. The PgR-levels before storage ranged between 75 and 230 fmoles/mg protein and became undetectable in all tumors (n=6) under these storage conditions. Thus it appears that both ER and PgR proteins deteriorate during prolonged storage at -70°C, but estradiol receptor proteins resist these conditions better than progesterone receptor proteins.

2.7.2 Storage of human breast tumor specimens in liquid nitrogen

Residue tumor specimens were re-analysed after varying storage periods in liquid nitrogen ranging between 2 and 18 months. Before storage the ER levels ranged between 16 and 606 fmoles/mg protein. In 16 of 17 tumors analysed the quantitative values observed after storage in liquid nitrogen were not different from the corresponding values measured before. The remaining tumor had an ER value of 60 fmoles/mg protein and showed no ER binding 14 months later. In 14 tumors PgR levels were determined before and after storage in liquid nitrogen. Before storage the values ranged between 70 and 1120 fmoles/mg protein. After storage 4 tumors showed no PgR binding, in 3 tumors the levels had declined 50% or more and in 7 tumors the levels did not change.

These observations indicate that, as far as the progesterone receptor is concerned, in some tumors receptor activity is not fully preserved even under conditions of this very low temperature.

2.7.3 Storage of lyophilised target tissues; a standard preparation suitable for interlaboratory quality control

Comparison of assay techniques of different laboratories has not been without difficulty because of the lability of receptor proteins upon storage (2.7.1 and 2.7.2) and the heterogeneity of breast tumor specimens (Rao and Meyer, 1977). Therefore the effect of lyophilisation and subsequent storage at 4°C or at room temperature on ER- and PgR-binding sites of a number of target tissues was investigated. The results were recently published (Koenders et al., 1978) and can be summarized as follows:

- during lyophilisation of DMBA-induced rat mammary tumor tissues, human breast tumor tissues and calf uterus tissues no deterioration of ER and PgR occurred.
- lyophilisation did not influence dissociation constants (K_d) or steroid specificities of ER and PgR binding sites.
- no loss of ER and PgR was observed when lyophilised calf uterus tissue was stored for 5 months at 0 - 4°C (Fig. 2.17) and for 3 weeks at room temperature.

Recently the effect of storage of lyophilised human breast tumor tissue was investigated. It appeared that no or only a minor decrease of ER binding sites occurred during storage at 0 - 4°C for 4 months or at room temperature for at least nine days. During storage of this tissue at 0 - 4°C no loss of PgR was observed during the first 35 days. Thereafter the PgR binding declined slowly ($t_{\frac{1}{2}}$ = 30 days). Storage of this tissue at room temperature showed that after 28 days the PgR level had decreased 60%.

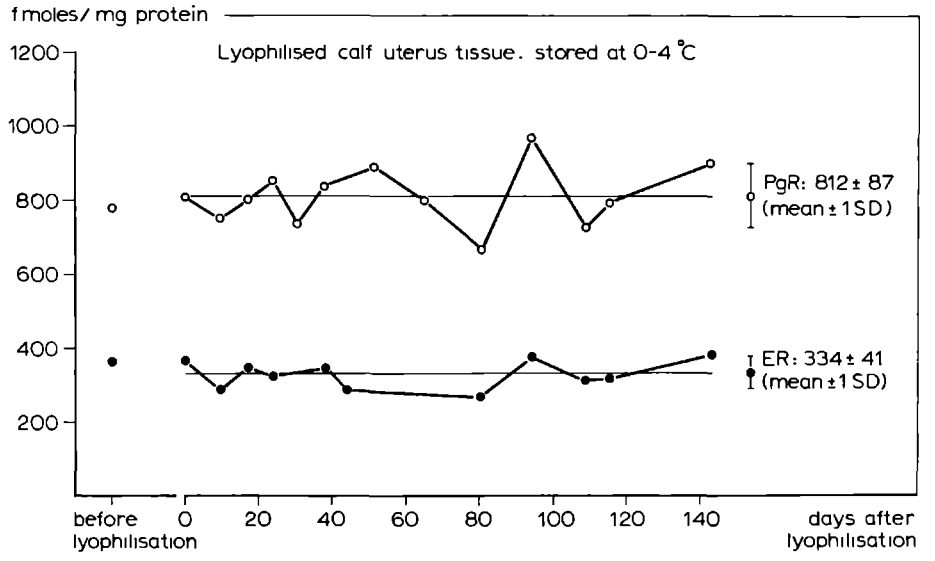


Fig. 2.17 Pulverized calf uterus tissue was lyophilised in glass vials and stored at 0 - 4°C. On various days lyophilised tissue powder has been analysed for the presence of ER and PgR.

It is generally accepted that patients with estradiol receptor-negative metastatic breast tumors have a poor chance to respond to endocrine therapy. Currently, in many clinics, when a tumor has been assayed as estradiol receptor-negative, the patients are excluded from endocrine treatment and are subjected to chemotherapy.

It is obvious, that when a negative result of an assay has such far reaching consequences, the laboratory has a need for explicit criteria by which a tumor can be labeled as receptor-positive or receptor-negative.

In this chapter attention has been paid to the evaluation of the receptor binding data especially at the lower limit of sensitivity (2.3.2). Especially at this detection limit Scatchard analysis is highly useful to distinguish receptor-positive and receptor-negative tumors. From the Scatchard plot it can be derived whether high affinity binding sites (K_d<4 nM) are present.

In this study the lowest receptor values actually measured in human breast tumor cytosols were 5 and 8 fmoles/mg protein for ER and PgR respectively. From cytosol dilution experiments (2.3.2) it appeared that ER values became underestimated, when the protein concentration becomes smaller than 2 mg/ml cytosol. It has been shown that below this protein concentration human breast tumor cytosols may even be falsely scored as ER-negative (Table 2.4). Therefore it may be recommended in the case of receptor-negative tumors to add information about the protein content of the cytosol. Actually a sharp decline in the incidence of ER-positive tumors was observed when the protein concentration of the cytosol was smaller than 2 mg/ml.

It has been suggested that the estradiol receptor level may be overestimated in the presence of contaminating Sex Hormone Binding Globulin (SHBG) (Ratajczak and Hähnel, 1976; Sherman and Miller, 1976; Raynaud et al., 1978). Whether SHBG actually interferes with the estradiol receptor assays, performed in human breast tumors, has been evaluated (2.5.2). Estrogenic compounds such as diethylstilbestrol or moxestrol, have a high affinity for the estradiol receptor (2.5.1) but hardly bind to SHBG. Therefore these compounds have been used for the assessment of nonspecific binding and the results were compared with those obtained using excess of radioinert estradiol itself (2.5.2.4).

It appeared that, if present, the interference was slight and occurred only in tumors showing relatively low receptor concentrations. This finding was to a large extent confirmed in experiments in which ^{3}H-moxestrol was used as the radioactive ligand and in experiments in which the contact time of the cytosol with dextran-coated charcoal was increased from 10 to 90 min. Okret et al. (1978) developed an isoelectric focusing assay in which ER and SHBG proteins are separated. They concluded that the degree of serum contamination in tumor specimens obtained as surgical biopsies does not interfere with the assay of ER. Furthermore these investigators concluded that ^{3}H-moxestrol eliminates SHBG interference when in tissue specimens, such as collected by fine needle aspiration, excessive serum contaminations were present. Another group of investigators, for other reasons, also recommended the use of ^{3}H-moxestrol as the radioactive ligand in the assay of ER in human breast tumors (Raynaud et al., 1978). Raynaud et al. (1978) observed that several tumors were receptor-positive using ^{3}H-E_2 and receptor-negative using ^{3}H-moxestrol and concluded that these tumors could in fact be merely contaminated by plasma. It has to be emphasized however, that in 4 out of 6 tumors which in our study had to be classified as receptor-positive using ^{3}H-E_2 and as receptor-negative using ^{3}H-moxestrol, progesterone receptor activity was present. This finding strongly indicates that the ER binding sites measured do not represent SHBG interference.

The steroid specificity of estradiol receptor binding sites was established by competition analysis, using ^{3}H-E_2 as the radioactive ligand, and by direct binding studies using several radioactive estrogenic compounds (2.5.1). Interestingly, remarkably discrepancies were observed between the K_d-values obtained by direct binding studies as compared to those obtained by competitive experiments. These results are in accordance with those reported by Rochefort and Capony (1977), Nicholson et al. (1977), Capony and Rochefort (1978) and Okey and Bondey (1978) for several other estrogenic and antiestrogenic compounds.

In this as well as in several other studies it was observed that K_d-values for steroid hormone receptor complexes show a wide range, when human breast tumors are analysed (Braunsberg et al., 1975; Leclercq et al., 1973). The determination of the K_d-values in these type of assays occurs under non-equilibrium conditions.

Under these conditions, in which tracer is stripped from low affinity binding sites (2.5.2.3), the free ligand concentration is overestimated giving Scatchard plots in which the slope becomes less steep, whereas the intercept on the abscissa is correct (Blondeau and Robel, 1975). This may explain why the addition of albumin or serum proteins to a calf uterus cytosol (2.5.2) increased the K_d-values, but had no influence on the estimation of ER binding sites.

For the assay of PgR a synthetic progestin, ^{3}H-R5020, was used as the radioactive ligand. This compound was recommended because it has high affinity for the progesterone receptor and hardly binds to CBG (Raynaud, 1977). In addition its complex with the progesterone receptor dissociates more slowly as compared to progesterone itself. The assay for PgR was evaluated by comparing the results obtained with this ligand and those obtained using another synthetic progestin, ^{3}H-Org 2058. It has been reported that Org 2058 binds to plasma proteins to a lower extent than R5020 (Jänne et al., 1976). The PgR values actually measured with either radioactive ligand did not show systematic differences. This finding does not support the suggestion of Seematter et al. (1978) that R5020 binding to human serum albumin interferes with the assay of PgR.

When estradiol receptor assays were introduced in 1971 it was believed adequate to distinguish between receptor-positive and receptor-negative tumor specimens (Jensen et al., 1971). From the first reports it appeared that approximately 50% of the breast tumors contained specific estradiol receptors (Wittliff, 1974). However since that time the reported incidence of ER-positive tumors has risen to even as high as 70-85% (McGuire, Carbone and Vollmer, 1975). This rise has been attributed to increased sensitivity of the methods employed. Several recent studies have suggested that quantitative ER values can be used to distinguish between patients which have a high or a low probability in obtaining a remission to endocrine therapy (DeSombre and Jensen, 1977; Engelsman, 1978). Because there is considerable variation between different assay methods and values obtained, there is a serious need for inter-laboratory quality control.

Recently for the ER assay a quality control study has been reported (King et al., 1978). In this study frozen breast tumor pieces have been used. It appeared that, although most laboratories with a few exceptions agreed whether a tissue specimen was ER-positive or ER-negative, remarkable differences in quantitative ER levels were obtained.

These differences were apparently related to the method of homogenisation, the presence or absence of thiol reagens and tumor heterogeneity.

In the present study it was shown that the ER and PgR levels of target tissues were not influenced by lyophilisation (2.7.3). Lyophilised tissues can be stored at 0 - 4°C for at least one month without loss of receptor activity. Even storage performed at room temperature for 1-2 weeks showed no or only a minor decrease of ER and PgR binding sites. Therefore lyophilised tissue powder seems suitable for inter-laboratory quality control studies.

CHAPTER 3

ESTRADIOL AND PROGESTERONE RECEPTORS IN DMBA-INDUCED MAMMARY TUMORS BEFORE AND AFTER OVARIECTOMY AND AFTER SUBSEQUENT ESTRADIOL ADMINISTRATION.

3.1 Introduction

Breast cancers induced in rats by the administration of a polycyclic aromatic hydrocarbon 7,12-dimethyl benzanthracene (DMBA) often show striking growth responses to endocrine ablations and additive hormone treatment (Huggins and Yang, 1962; Griswold and Green, 1970; Pearson et al., 1972). The presence of estradiol receptors in these experimental tumors has been well documented (Terenius, 1968; McGuire and Julian, 1971; Leclercq and Heuson, 1973). Some authors claimed that estradiol receptor binding sites were selectively present in tumors which regressed after ovariectomy (McGuire and Julian, 1971; Nomura et al., 1974). More recently it has been reported that essentially all DMBA-tumors, ovarian-dependent as well as ovarian-independent, contain estradiol receptors (Boylan and Wittliff, 1975; DeSombre et al., 1976). In a small number of studies it has been attempted to relate the level of estradiol receptors to the responsiveness to ovariectomy of DMBA-tumors (Mobbs and Johnson, 1974; DeSombre et al., 1976). Although ER levels in ovarian-independent tumors tended to be lower than those in overian-dependent tumors, there was considerable overlap between the corresponding values obtained in the two groups of tumors.

It has been hypothesized by McGuire et al. (1975) that for the prediction of endocrine responsiveness of breast tumors, the progesterone receptor - being an end product of estrogen action - would be a better marker than the estradiol receptor which, according to these authors, is only involved in the initial step in estrogen action.

In the present study the relation between ER levels and endocrine responsiveness was re-examined. Furthermore it was analysed whether measurement of the progesterone receptor would give more reliable information about hormone-dependency of DMBA-tumors.

Finally the present study analyses whether estradiol - as in normal target tissues - exerts control over the production of the progesterone receptor in DMBA-tumors.

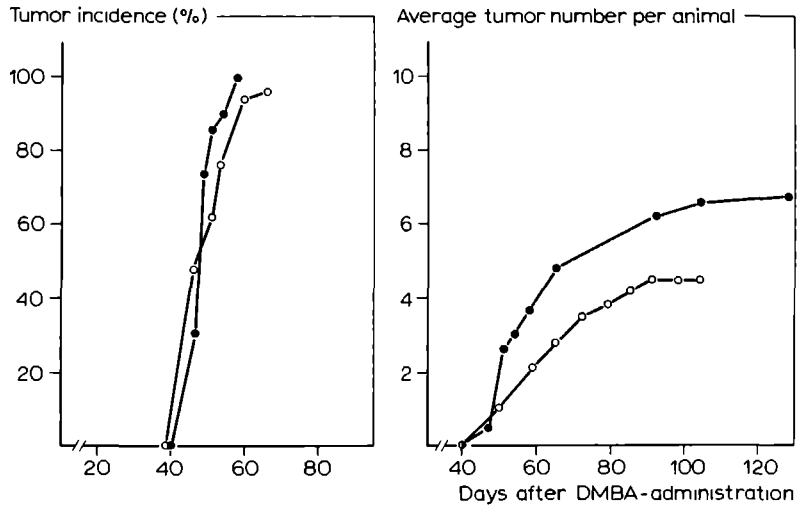
3.2 Experimental Procedures

3.2.1 Mammary tumor induction

Mammary tumors were induced in 50 days old female Sprague-Dawley rats by a single intragastric feeding of 20 mg DMBA in 1.0 ml sesame oil (Huggins et al., 1961) . The animals used for the first experiment (described in this chapter) were obtained from the Zentralinstitut für Versuchstierzucht, Hannover/Linden (W. Germany). In the first two weeks after DMBA-administration 15% (9/60) of the animals died. Autopsy revealed similar abnormalities as described by Huggins and Morii (1961) and Dao (1964) e.g. leukopenia, diminished food intake, gastrointestinal tract bleeding, degenerative changes in liver, kidney and lymph nodes and enlarged adrenals, which were dark red in color. Huggins and Dao observed necrosis and hemorrhage of the adrenals and attributed the sudden death of these animals to adrenal apoplexy. The first mammary tumors were detected in the seventh week after DMBA-administration (Fig. 3.1). Thereafter all animals rapidly obtained mammary tumors and at day 58 each animal had at least one mammary tumor. The average number of tumors per animal reached a maximum of about 7, 120 days after DMBA-administration (Fig. 3.2). Some animals obtained as many as 10 tumors.

3.2.2 Assessment of mammary tumor growth and excision of tissue biopsies

Tumor areas of all tumors of each animal were calculated at least once every week from caliper measurements of two diameters at right angles (length x width; sq cm). The diameter of a tumor had to be about 0.7 cm (0.5 sq cm) in order to be precisely measured. When the first tumor reached an area of about 4 sq cm a small biopsy (0.5 - 1.0 g) was taken at random stages of the oestrus cycle under light ether anesthesia. As a consequence of this procedure, tumors were selected. Tumors with a very slow growth rate, tumors which remained small and tumors which developed after a long time were not included in the receptor studies. The excised biopsy specimen was washed in cold saline and trimmed of fat, connective tissue and necrotic tumor areas. The biopsy was cut into small pieces and a representative section was removed for histological examination. All tumors included in the receptor studies were classified as adenocarcinomas (Dr. K. Kubàt, Department of Pathology, University of Nijmegen).



Figs. 3.1 and 3.2 *Induction of mammary tumors in female Sprague-Dawley rats following a single feeding of 20 mg DMBA.*

Fig. 3.1 *Tumor incidence as a function of time after DMBA-administration.*

Fig. 3.2 *Average tumor number per animal as a function of time after DMBA-administration.*

● ——— ● *Sprague-Dawley rats; W. Germany (Chapter 3)*

○ ——— ○ *Sprague-Dawley rats; France (Chapter 4).*

The specimens were immediately frozen in liquid nitrogen and stored at -70°C in a Revco Ultra Deepfreezer. When tumors regained prebiopsy size, the animals were bilaterally ovariectomized under light ether anesthesia. After the effect of this treatment on the tumor growth was assessed a second biopsy was taken from the same tumor already biopsied before. The rate of tumor regression after the removal was generally very rapid and the biopsies were on the average excised 10 days after ovariectomy. Ovariectomized animals with regressed tumors subsequently received 5 µg estradiol as the benzoate (s.c.) daily. After the tumor growth response to estradiol administration was established the animals were sacrificed and the tumors were excised 24 hours

after the last estradiol injection.

3.2.3 Classification of mammary tumors

Fig. 3.3 shows examples of tumor types in respect to their responsiveness to ovariectomy and the subsequent replacement of estrogen, which were included in the receptor studies. Four major types of tumors were obtained:

1. Ovarian-independent tumors, which continued to grow after ovariectomy (Fig. 3.3a).
2. Ovarian-dependent tumors, which regressed at least 25% within 10 days after ovariectomy (Fig. 3.3b). Of all growing tumors 86% (55/64) were scored as ovarian-dependent and 14% (9/64) as ovarian-independent.
3. Estradiol-responsive tumors, which regressed after ovariectomy and regrew after subsequent estradiol administration (Fig. 3.3c).
4. Estradiol-unresponsive tumors, which regressed after ovariectomy, but did not regrow after estradiol administration. In some of these tumors regression stopped, when estradiol was administered, whereas in others regression continued (Fig. 3.3d). Of the tumors, which regressed after ovariectomy 40% (15/39) were classified as estradiol-responsive and 60% (24/39) as estradiol-unresponsive.

It has to be emphasized that in the same animal the tumors responded differently to endocrine treatment. It occurred that after ovariectomy one tumor showed growth accompanied by regression of others.

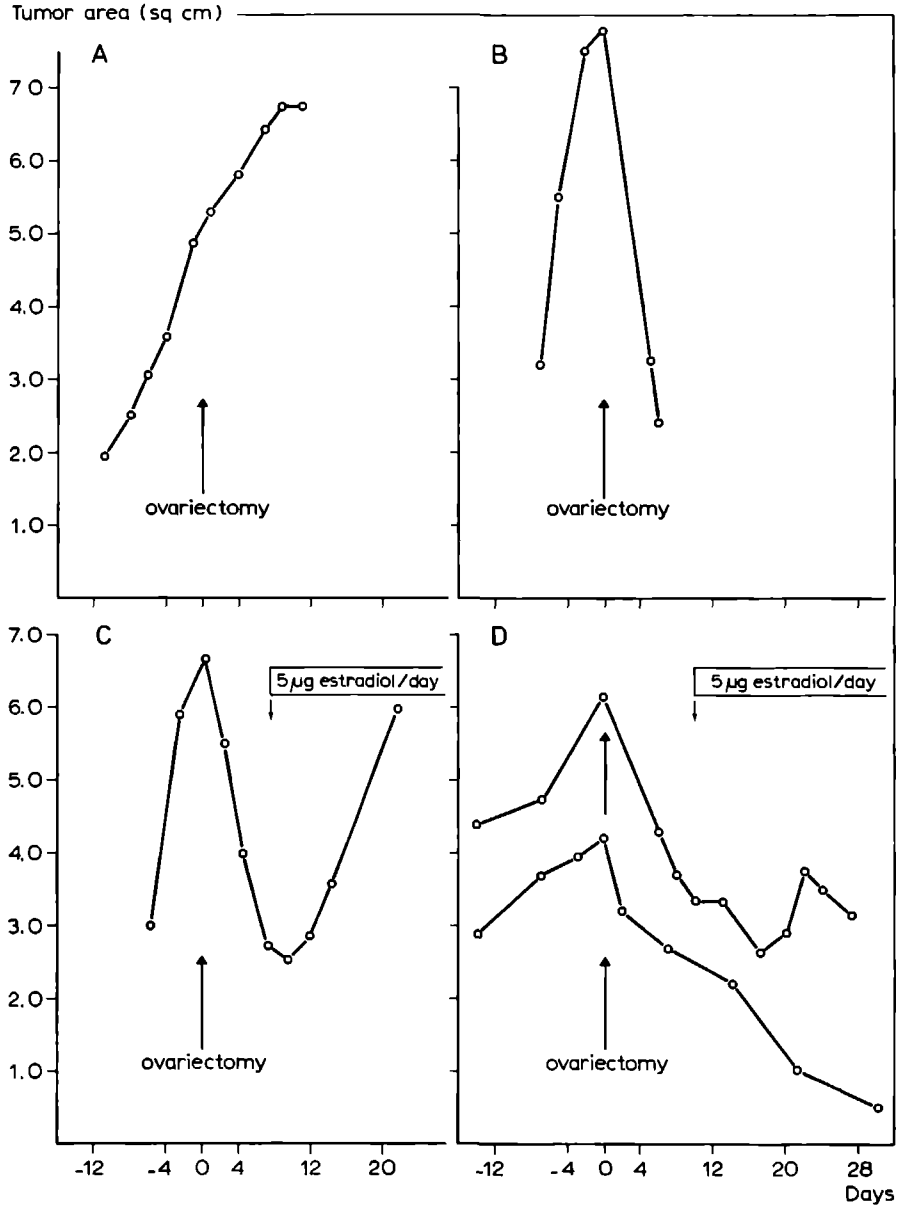


Fig. 3.3 Classification of DMBA-induced rat mammary tumors on the basis of growth response to ovariectomy and subsequent estradiol administration.

A: ovarian-independent tumor ; B: ovarian-dependent tumor

C: estradiol-responsive tumor ; D: estradiol-unresponsive tumors.

3.3.1 Estradiol and progesterone receptor levels before ovariectomy in ovarian-dependent and ovarian-independent tumors.

In 20 from the 55 ovarian-dependent and in all 9 ovarian-independent tumors ER was measured. PgR was assayed in 16 of these 20 ovarian-dependent and in 7 of the 9 ovarian-independent tumors. Representative Scatchard plots from 6 points assays are presented in Fig. 3.4. The uncorrected plots were not linear indicating that both ^{3}H-E_2 and ^{3}H-R5020 were bound to at least two different binding proteins (see also chapter 2.3.1). After correction for nonspecific binding linear plots were obtained with low K_d-values. The K_d-values obtained were similar in ovarian-dependent and ovarian-independent tumors: K_d for ER 0.32 $\pm$ 0.03 (n=20, dependent) and 0.40 $\pm$ 0.05 (n=9, independent), K_d for PgR 2.9 $\pm$ 0.4 (n=16, dependent) and 2.4 $\pm$ 9 nM (n=5, independent). The steroid specificity of ER and PgR binding sites did not differ for ovarian-dependent and ovarian-independent tumors. ER was present in all tumors, the levels ranged from 15 - 305 in ovarian-dependent and from 10 - 80 fmoles/mg protein in ovarian-independent tumors (Table 3.1). Although there was no sharp distinction between dependent and independent tumors, the ER levels of the dependent tumors were significantly higher than those of the independent tumors ($p<0.01$, Wilcoxon rank test).

Table 3.1 also shows that, in spite of ovarian-independency 5 out of 7 tumors contained PgR binding sites. The PgR levels ranged from 45 - 385 in ovarian-dependent and from 0 - 185 fmoles/mg protein in the ovarian-independent tumors, showing again a considerable overlap. Statistically however the PgR levels in ovarian-dependent tumors were significantly higher than in ovarian-independent tumors ($p<0.05$, Wilcoxon rank test).

The relation between both the ER and PgR levels in the ovarian-dependent and ovarian-independent tumors is illustrated in Fig. 3.5. In the dependent tumors the correlation coefficient between these levels was significant ($r_{Spearman} = 0.61$, $p<0.02$). No such correlation was found in the independent tumors ($r_{Spearman} = 0.05$, $p>0.1$).

Analogous to studies of other investigators of hormone-dependency of tumors arbitrarily chosen "critical levels" (25 fmoles/mg protein for ER and 55 fmoles/mg protein for PgR) have been drawn in Fig. 3.5 (DeSombre et al, 1976; Jensen et al., 1975). All tumors with both receptor levels above these "critical levels" belonged to the group of ovarian-dependent tumors.

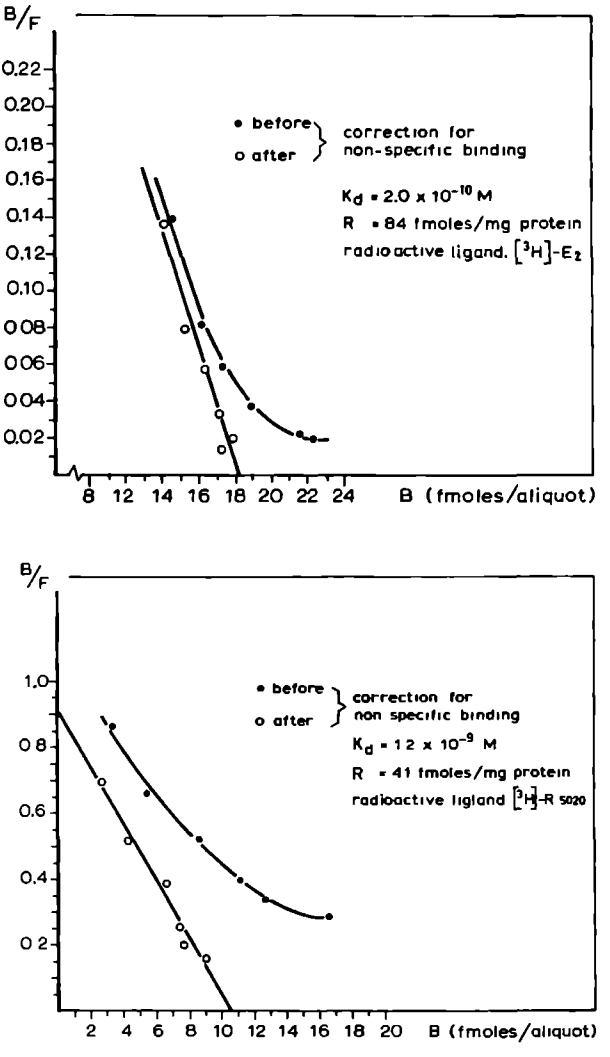


Fig. 3.4 Scatchard plots of mammary tumor cytosol incubated with ^{3}H-E_2 or ^{3}H-R5020. The Scatchard plots of the total binding (•) were corrected for nonspecific binding to obtain the Scatchard plots of specific binding (O).

Table 3.1 Estradiol and progesterone receptor levels before ovariectomy in ovarian-dependent and ovarian-independent mammary tumors.

ER (fmoles/mg protein)		PgR (fmoles/mg protein)	
ovarian-dependent tumors	ovarian-independent tumors	ovarian-dependent tumors	ovarian-independent tumors
15	10	45	0
25	10	45	0
35	15	60	20
40	15	75	55
50	15	75	55
50	20	80	100
60	25	85	185
65	35	110	
75	80	125	
75		130	
95		185	
100		205	
100		230	
100		275	
110		285	
135		385	
160			
175			
175			
305			
97 ± 15[a]	25 ± 7★★	149 ± 25	60 ± 25★
(n=20)	(n=9)	(n=16)	(n=7)

[a]Mean ± S.E.

★★$p<0.01$ (ovarian-dependent vs. ovarian-independent, Wilcoxon rank test)

★$p<0.05$ (ovarian-dependent vs. ovarian-independent, Wilcoxon rank test).

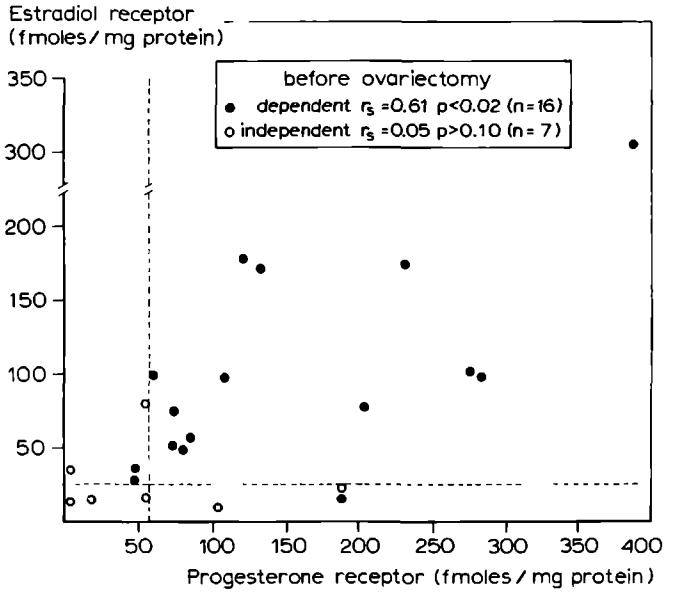


Fig. 3.5 Relation of pretreatment progesterone and estradiol receptor levels of ovarian-dependent (●) and ovarian-independent (O) DMBA-induced rat mammary tumors. Broken lines indicate arbitrarily chosen "critical levels".

On the other hand 3 tumors with ER and PgR levels below these "critical levels" were ovarian-independent. From the remaining 7 tumors with either ER or PgR levels below one of the "critical levels" 3 were ovarian-dependent and 4 ovarian-independent.

3.3.2 Estradiol and progesterone receptor levels before ovariectomy in estradiol-responsive and estradiol-unresponsive tumors

It is well known that most but not all tumors that regress after ovariectomy regrow after estradiol administration (Nicholson and Golder, 1975; Bradley et al., 1976). Often tumors are considered as hormone-responsive or hormone-dependent only, when regrowth after estradiol stimulation occurs. This to ensure that the regression was due to removal of endogenous hormone and was not spontaneous which occurs in these experimental tumors (Young and Cowan, 1963).

Table 3.2 Pre-ovariectomy ER and PgR levels in estradiol-responsive and estradiol-unresponsive tumors.

Estradiol-responsive		Estradiol-unresponsive	
ER	PgR	ER	PgR
(fmoles/mg protein)		(fmoles/mg protein)	
60	85	15	185
75	75	25	45
75	205	35	45
100	285	50	80
175	230	50	75
175	125		
110 ± 21[a]	165 ± 85	35 ± 7	87 ± 26
(n=6)	(n=6)	(n=5)	(n=5)

[a]Mean ± S.E.

Therefore the group of tumors which regressed after ovariectomy was subdivided: tumors were classified as estradiol-responsive when regrowth occurred after estradiol treatment and as estradiol-unresponsive when no regrowth was observed.

Estradiol-responsiveness was assessed in 11 out of the 16 ovarian-dependent tumors in which both ER and PgR levels had been measured (Table 3.1). Six of these 11 tumors appeared estradiol-responsive. The pre-ovariectomy ER and PgR levels of both estradiol-responsive and estradiol-unresponsive tumors are collected in Table 3.2. ER values in the estradiol-responsive tumors were all higher than the values observed in the estradiol-unresponsive tumors. The PgR levels of both groups of tumors showed a considerable overlap. Tumor regrowth occurred with estradiol administration in those tumors that had relatively high pre-ovariectomy ER and PgR levels.

It may be noted that the 3 ovarian-dependent tumors with ER or PgR values falling below one of the "critical levels" drawn in Fig. 3.5 belonged to the estradiol-unresponsive group.

3.4. Effect of ovariectomy and of estradiol administration on estradiol and progesterone receptor levels

3.4.1 Effect of ovariectomy on estradiol and progesterone receptor levels

Although after ovariectomy the estradiol receptor levels of ovarian-dependent tumors on the average, declined to approximately 50% of the pretreatment values (Fig. 3.6), in 9 of the 18 tumors the values did not decrease. In 6 out of 7 ovarian-independent tumors the receptor levels did not change essentially after ovariectomy. The PgR levels of ovarian-dependent and ovarian-independent tumors, before and after ovariectomy are shown in (Fig. 3.7). Ovariectomy strikingly decreased PgR levels in both dependent and independent tumors. It seems worth mentioning that the highest three post-ovariectomy values of the ovarian-dependent tumors were obtained in biopsies taken on days 3, 4 and 7 after ovariectomy. From both Fig. 3.6 and 3.7 it follows that after ovariectomy PgR in most tumors became undetectable, despite the preservation of ER.

3.4.2 Effect of estradiol administration on estradiol and progesterone receptor levels

After assessing ovarian dependency of the tumors 5 μg estradiol as the benzoate (s.c. daily) was administered for 2 to 4 weeks.

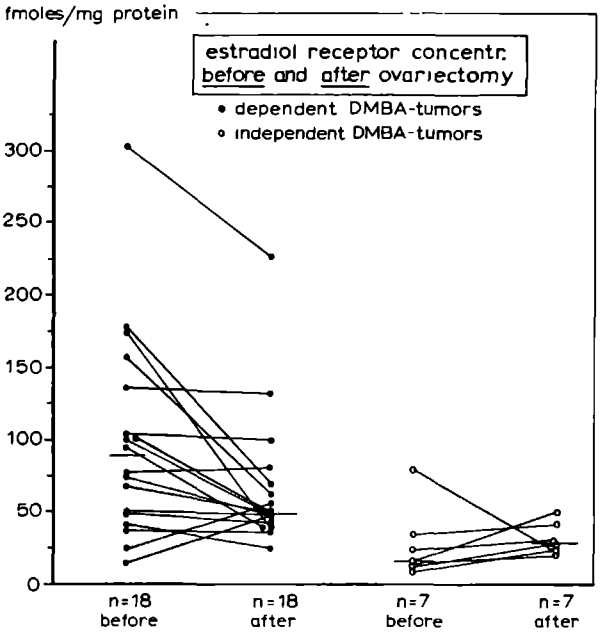


Fig. 3.6
Effect of ovariectomy on estradiol receptor levels of ovarian-dependent (●) and ovarian-independent (○) DMBA-induced rat mammary tumors. Median values are indicated by horizontal lines.

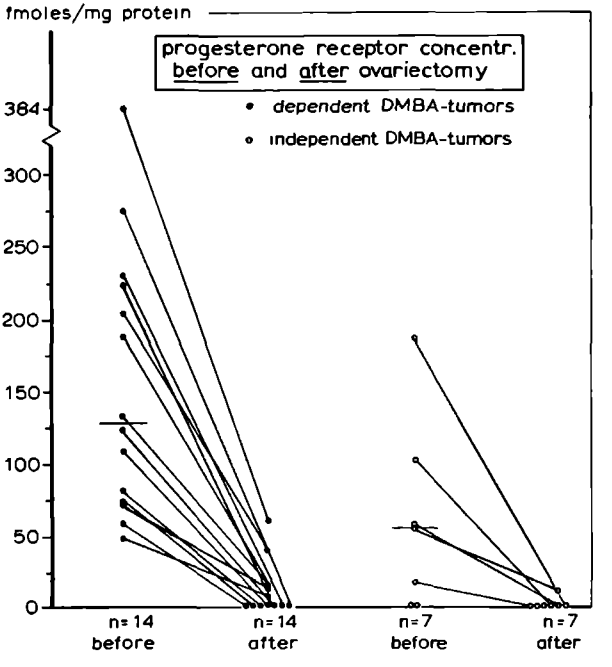


Fig. 3.7
Effect of ovariectomy on progesterone receptor levels of ovarian-dependent (●) and ovarian-independent (○) DMBA-induced rat mammary tumors. Median values are indicated by horizontal lines.

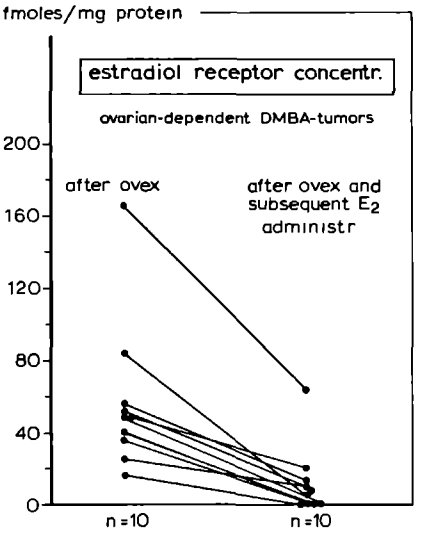


Fig. 3.8
Effect of estradiol administration to ovariectomized animals on estradiol receptor levels of ovarian-dependent DMBA-induced rat mammary tumors.

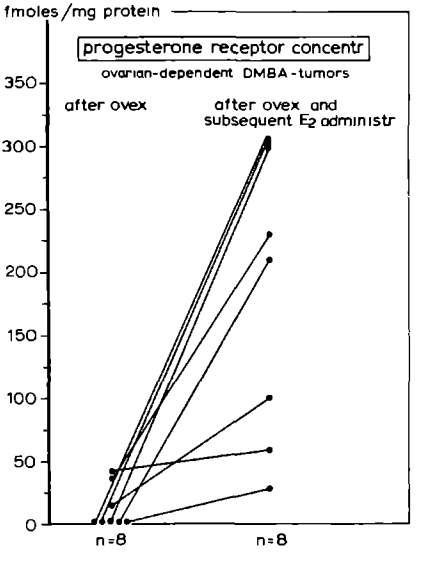


Fig. 3.9
Effect of estradiol administration to ovariectomized animals on progesterone receptor levels of ovarian-dependent DMBA-induced rat mammary tumors.

Estradiol receptor levels decreased to very low or undetectable levels during this long term treatment (Fig. 3.8). This decrease might be explained by the relatively high dose of estradiol benzoate administered. This could have caused occupation of cytoplasmic receptor sites and/or translocation of estradiol receptor complexes to the nucleus.

It is of interest that ER levels also decreased in the uteri of these tumor bearing animals, but never became undetectable. The mean $\pm$ S.E. of the uterine ER levels after ovariectomy and after estradiol administration to ovariectomized animals was 420 $\pm$ 60 (n=6, range 255 - 620 fmoles/mg protein) and 75 $\pm$ 10 (n=9, range 40 - 110 fmoles/mg protein), respectively.

The PgR levels of ovarian-dependent tumors, which were analysed before and after estradiol are shown in Fig. 3.9. In all tumors (n=8), which regressed after ovariectomy, estradiol treatment increased PgR levels. It is worth mentioning that during estradiol administration all tumors (n=8) showed PgR increase, whereas 4 tumors did not regrow. The PgR levels of ovarian-dependent tumors before ovariectomy (149 $\pm$ 25, n=16) and of dependent tumors of ovariectomized animals receiving estradiol (191 $\pm$ 40 fmoles/mg protein, n=8) were not different ($p>0.1$, Wilcoxon rank test).

3.5 Discussion

All DMBA-tumors analysed in the present investigation contained detectable amounts of ER. Therefore it is impossible to distinguish ovarian-dependent from ovarian-independent tumors solely on the presence or absence of ER, a possibility, which has been claimed by some authors (McGuire and Julian, 1971; Nomura et al., 1974), but recently denied by others (Mobbs and Johnson, 1974; Boylan and Wittliff, 1975; DeSombre et al., 1976). It is of particular interest that pre-ovariectomy ER levels of the ovarian-dependent tumors were significantly higher than the ER levels of the ovarian-independent tumors. Eighteen out of 20 ovarian-dependent tumors had receptor levels >25 fmoles/mg protein, whereas 7 out of 9 independent tumors showed levels equal to or below this value.

It may be noted that in the present study biopsies of the tumors were taken before ovariectomy, whereas in other investigations, which also attempted to relate the level of ER to ovarian-responsiveness of DMBA-tumors, ER levels were measured after the respons to ovariectomy was assessed (Boylan and Wittliff, 1975; DeSombre et al., 1976).

In that case several weeks may have relapsed between ovariectomy and excision of the tumor for receptor analysis, a procedure which, as ovariectomy may have a pronounced effect on ER levels, might not be preferable.

It may be stressed that among the ovarian-dependent tumors a small number showed low pre-ovariectomy ER values indicating that there is no straight forward relation between estrogen binding capacity of a tumor and its growth response to ovariectomy as was also observed by other investigators (Mobbs and Johnson, 1974; Boylan and Wittliff, 1975; DeSombre et al., 1976).

Results from this investigation demonstrate that ovarian-independent tumors can contain significant amounts of cytoplasmic progesterone receptor. The PgR levels in 5 out of 7 independent tumors ranged from 20 - 185 fmoles/mg protein. This finding does not support the attractative hypothesis of McGuire et al. (1975) regarding PgR as an ideal marker of hormone dependence. McGuire et al. (1975) reasoned, "that the presence of the progesterone receptor in ER-positive tumors would indicate that the tumor is capable of synthesizing atleast one end product under estrogen regulation and may therefore be endocrine-responsive". Recently these authors emphasized that a number of observations (e.g. in a tumor cell line, which growth appeared to be estrogen-independent, PgR was estrogen controlled) indicate that effects of estrogens on growth and on PgR might well be dissociated (Horwitz and McGuire, 1978). As will be discussed later some results of the present study are in accordance with this conclusion.

At the time this investigation was started it was unknown whether PgR in DMBA-tumors is under acute control of estradiol as in normal target tissues. The results presented in this chapter strongly suggest - in accordance with recent reports - that indeed PgR is controlled by estradiol in the malignant transformated cells of DMBA-tumors: ovariectomy caused a precipitous fall in PgR levels to very low or undetectable levels, whereas estradiol administration after ovariectomy induced a pronounced increase in PgR levels (Tsai and Katzenellenbogen, 1977; Horwitz and McGuire, 1977; Kelly et al., 1977; Pearson et al., 1977; Asselin and Kelly, 1978). It is noteworthy that the fall in PgR levels after ovariectomy was not restricted to ovarian-dependent tumors but was also observed in ovarian-independent tumors. This observations points to the above mentioned dissociation between estradiol responsiveness and estradiol induced PgR synthesis. Another observation is in line with such dissociation: the increase in PgR levels in a number of tumors, induced by estradiol administration, did not coincide with

regrowth of the tumor. These results suggest that in DMBA-tumor cells PgR synthesis remains estradiol controlled even when the growth of the tumor has become estrogen-independent.

Some authors (McGuire and Julian, 1971; Mobbs and Johnson, 1974) consider a tumor as being unequivocally hormone-dependent only when regression of the tumor occurs upon ovariectomy and a regrowth is observed when estradiol is administered subsequently. When these criteria would have been used in the present investigation, the unequivocal hormone-dependent tumors all had relatively high estradiol and relatively high progesterone receptor levels.

In the present study it was found that measurement of ER or PgR levels were of equal value to predict ovarian-dependency or -independency of DMBA-induced rat mammary tumors. Nevertheless absolute discrimination between both groups of tumors could not be reached by either parameter or both. Several possibilities can be considered why more adequate predictability of ovarian-dependency had not been reached. First, as has recently been reported, levels of ER, and this certainly may also hold for PgR, fluctuate during the oestrus cycle (Hawkins et al., 1977; Shih and Lee, 1978), a fact which has not been taken into account in the present study. Second, it is possible that other hormones are also involved in the growth regulation of DMBA-induced breast cancer. A serious candidate in this respect is the peptide hormone prolactin. There is a growing body of evidence that prolactin acts in concert with estradiol in regulating tumor growth in these experimental tumors (Leung, 1978). Third, receptor levels in this study are related to the protein content of the cytosol, that means that not only tissue protein, but also plasma proteins are involved. It might be preferable to express the receptor binding capacity on the basis of DNA content.

CHAPTER 4

ESTRADIOL AND PROGESTERONE RECEPTORS IN DMBA-INDUCED MAMMARY TUMORS BEFORE AND DURING TAMOXIFEN ADMINISTRATION.

4.1 Introduction

Antiestrogens are a class of compounds which prevent estrogens from expressing their full effect on estrogen target tissues (Katzenellenbogen and Ferguson, 1975; Burnett Lunan and Klopper, 1975). Although the mechanism of action of antiestrogens is not clearly defined, it is generally accepted that an interaction with the estradiol receptor is an essential step. Antiestrogens compete with estradiol for the cytoplasmic estradiol receptor and inhibit the uptake of labeled estradiol by estrogen-sensitive target tissues e.g. uterus and vagina (Rochefort and Capony, 1972; Terenius and Ljungkvist, 1972). Antiestrogens are capable of stimulating the translocation of estradiol receptors from the cytoplasm to the nucleus, which results in a prolonged depletion of cytoplasmic estradiol receptors (Ruh and Ruh, 1974; Capony and Rochefort, 1975; Katzenellenbogen and Ferguson, 1975; Jordan et al., 1977). Clarke et al. (1973) have suggested that antiestrogens may produce their antagonistic effects by inhibiting the resynthesis of cytoplasmic estradiol receptors.

Antiestrogens are widely used in the palliative treatment of advanced breast cancer, mainly in postmenopausal patients (Cole et al., 1971; Ward, 1973; Heuson, 1976). Furthermore it has been reported that antiestrogens inhibit the growth of DMBA-induced rat mammary tumors (Nicholson and Golder, 1975; Jordan and Koerner, 1975; Jordan, 1976). There is ample evidence that also in these tumors the action of antiestrogens is directly mediated by the estradiol receptor system (Nicholson et al., 1976; Nicholson et al., 1977 II; Jordan and Koerner, 1975). Two recent studies suggested a relation between growth response of DMBA-tumors to the antiestrogen tamoxifen and the estradiol receptor levels (Jordan and Jaspan, 1976; Nicholson et al., 1977). No information is available with regard to the progesterone receptor.

In the present study the relation between pretreatment ER as well as PgR levels and tumor response to tamoxifen administration was investigated. In addition the effect of this treatment on ER and PgR levels was studied. In the course of this investigation it appeared that during tamoxifen admini-

stration tumor growth became autonomous. It seemed of interest to determine the presence of ER and PgR in these autonomous tumors and particularly whether the synthesis of PgR, if present, was still under estrogen control.

4.2 Experimental procedures

4.2.1 Tamoxifen treatment and excision of tumor biopsies

Induction of DMBA-tumors was described in 3.2.1. In this experiment Sprague-Dawly rats of a different strain (IFFA Credo, France) were used, of which it was reported that only one to three mammary tumors appear after the administration of 20 mg DMBA (Vignon and Rochefort, 1976). Twenty-nine percent (22/75) of these animals died within two weeks after DMBA-administration. From Fig. 3.1 it can be seen that the rate of appearance of mammary tumors was similar in both strains of animals. The average number of mammary tumors per rat was somewhat lower than in the first experiment, but still reached the value of 4.5 (Fig. 3.2).

Biopsies before tamoxifen administration were taken of tumors that reached an area of about 4 sq. cm (see 3.2.2). When tumors regained prebiopsy size the animals received daily s.c. injections of 200 µg tamoxifen in 0.1 ml sesame oil. After 3 weeks of tamoxifen treatment a second biopsy was taken from the same tumor already biopsied before. Thereafter tamoxifen treatment was continued until the animals had multiple rapidly growing tumors About two weeks after tamoxifen withdrawal biopsies of these growing tumors were taken.

4.2.2 Classification of mammary tumors

Regression occurred more slowly during tamoxifen administration in this study than after ovariectomy, described in the previous chapter. Therefore tumors were followed for 3 weeks before tumor response could be assessed. Growth patterns of tumor types included in the receptor studies are presented in Fig. 4.1. Tumors that had decreased at least 50% in size during 3 weeks of tamoxifen administration were scored as regressing tumors (Fig. 4.1a), whereas tumors that remained stable or continued to grow were scored as non-regressing tumors (Fig. 4.1b).

In a preliminary experiment the effect of 50 and 200 µg tamoxifen daily

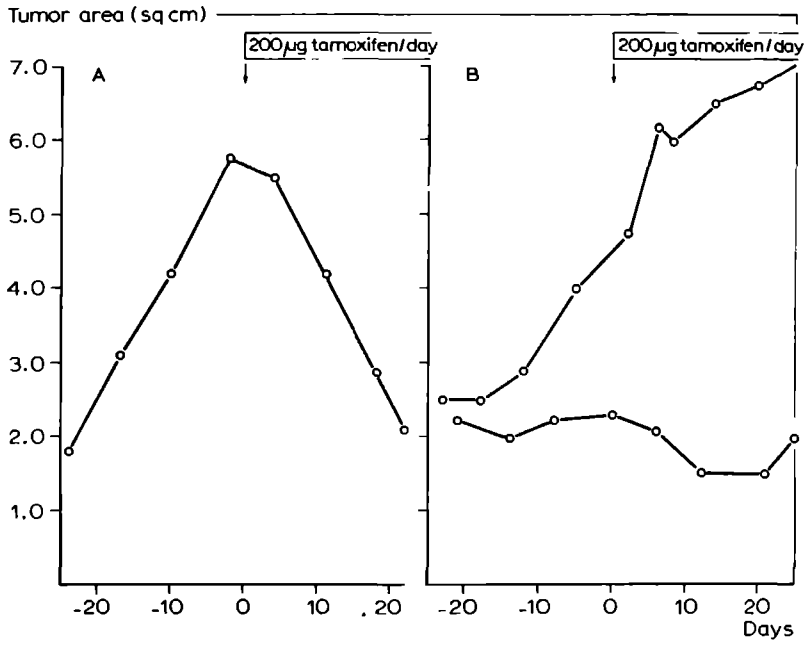


Fig. 4.1 Growth patterns of representative DMBA-induced rat tumors during 200 µg tamoxifen treatment.
A: regressing tumor
B: non-regressing tumors.

on tumor growth was studied. Table 4.1 shows that after 3 weeks of 50 µg tamoxifen treatment 22% of these DMBA-tumors appeared to be regressing, whereas after 3 weeks of 200 µg tamoxifen 70% of the tumors were scored as regressing ($p<0.001$). In the receptor studies only tumors of animals are included, which received 200 µg tamoxifen daily.

Table 4.1 Mammary tumor growth response to three weeks of tamoxifen treatment.

Treatment	no of rats	no of tumors	Regressing tumors	Non-regressing tumors
50 µg tamoxifen/day	12	50	11 (22%)★	39 (78%)
200 µg tamoxifen/day	12	47	33 (70%)	14 (30%)

The tumor responses have been assessed after 3 weeks of tamoxifen treatment. Tumors regressing to 50% of their pretreatment size were scored as regressing tumors.
The remaining tumors that remained static or grew despite this treatment were scored as non-regressing tumors.
★χ^2 = 20.8; DF = 1; $p<0.001$ (50 µg vs 200 µg tamoxifen).

Table 4.2 Estradiol and progesterone receptor levels in simultaneously taken biopsies from different tumors of the same animal.

Animal	ER (fmoles/mg protein)	PgR	tumor classification
1 tumor A	50	670	regressing tumor
tumor B	75	670	regressing tumor
2 tumor A	20	80	regressing tumor
tumor B	95	130	regressing tumor
3 tumor A	75	170	regressing tumor
tumor B	35	0	non-regressing tumor
4 tumor A	35	45	non-regressing tumor
tumor B	60	145	non-regressing tumor

4.3 Estradiol and progesterone receptor levels before tamoxifen treatment in regressing and non-regressing tumors

ER and PgR were measured in 24 tumor biopsies (19 animals) taken before 200 µg tamoxifen treatment. Of 4 animals two biopsies have been simultaneously taken from different tumors of the same animal (Table 4.2). It appeared that ER and PgR levels showed a wide range even in these tumor biopsies, that were simultaneously taken at the same stage during the oestrus cycle. Interestingly in animal no. 3 one of the tumors was scored as regressing and the other as non-regressing.

Table 4.3 shows the individual ER and PgR levels of regressing and non-regressing tumors. Detectable amounts of ER were found in 21 out of 24 tumors studied. The ER levels ranged from 0-95 in the group of the regressing tumors and from 0-60 fmoles/mg protein in the group of non-regressing tumors, thus showing a considerable overlap. Nevertheless the ER levels of the regressing tumors were significantly ($p<0.05$, Wilcoxon rank test) higher than those of the non-regressing tumors.

All regressing tumors (n=15) and 5 out of 9 non-regressing tumors revealed the presence of PgR. Although there was overlap of the lower values of the regressing tumors (range 40-900 fmoles/mg protein) and the higher values of the non-regressing tumors (range 0-255 fmoles/mg protein), regressing tumors had significantly higher PgR levels than non-regressing tumors ($p<0.01$, Wilcoxon rank test).

The relation between ER and PgR levels of regressing and non-regressing tumors is shown in Fig. 4.2. Remarkably in this experiment a significant correlation between individual ER and PgR levels in both groups of tumors was not observed (regressing tumors: $r_{Spearman} = 0.37$, $p>0.1$ and non-regressing tumors: $r_{Spearman} = 0.11$, $p>0.1$). Using arbitrarily chosen "critical levels" of 20 fmoles/mg protein for the estradiol receptor and of 150 fmoles/mg protein for the progesterone receptor, it appeared that all tumors (11/11) with both receptor levels above these "critical levels" regressed during 3 weeks of 200 µg tamoxifen administration. On the other hand only 4 out of the 13 remaining tumors with either an ER value below and/or a PgR value below these "critical levels" regressed during this treatment ($\chi^2 = 9.41$; DF = 1; $p<0.01$). Three tumors contained ER (15-35 fmoles/mg protein) whereas no PgR was detectable and all 3 tumors failed to regress during tamoxifen. Interestingly in 2 tumors PgR activity was present whereas no ER was detectable.

Table 4.3 Estradiol and progesterone receptor levels before tamoxifen treatment in regressing and non-regressing tumors.

ER (fmoles/mg protein)		PgR (fmoles/mg protein)	
regressing tumors	non-regressing tumors	regressing tumors	non-regressing tumors
0	0	40	0
20	0	40	0
25	15	80	0
30	15	130	0
35	25	165	45
35	35	170	110
40	35	210	115
45	35	260	140
50	60	295	255
50		400	
50		515	
60		670	
75		670	
75		750	
95		900	
46 ± 6[a]	25 ± 6★	352 ± 72	74 ± 30★★
(n=15)	(n=9)	(n=15)	(n=9)

[a]Mean ± S.E.

★$p<0.05$ (regressing vs. non-regressing, Wilcoxon rank test)

★★$p<0.01$ (regressing vs. non-regressing, Wilcoxon rank test)

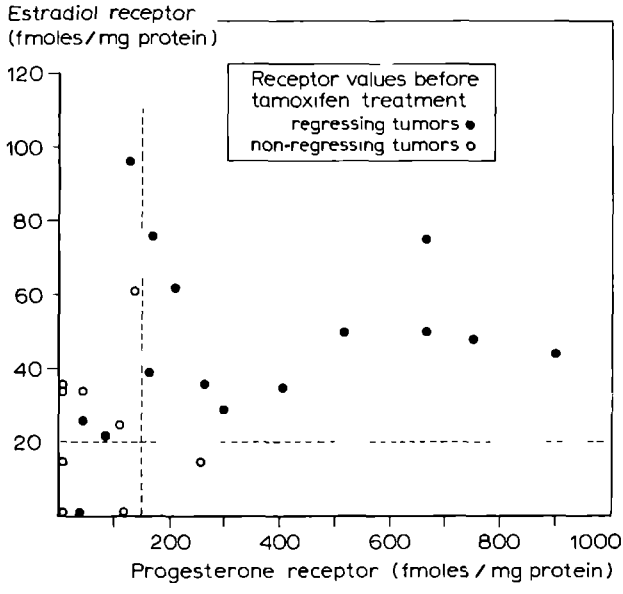


Fig. 4.2 Relation of pretreatment progesterone and estradiol receptor levels of regressing (•) and non-regressing (o) DMBA-induced rat mammary tumors.
Broken lines indicate arbitrarily chosen "critical levels".

4.4 Estradiol and progesterone receptor levels before and during tamoxifen administration

Biopsies of the same tumor were taken before and during tamoxifen administration. The animals received 200 µg tamoxifen for approximately 3 weeks and the tumors were assayed 48 hrs after the last injection. Fig. 4.3 compares ER and PgR levels of the same tumor before and during tamoxifen treatment. During tamoxifen treatment estradiol receptors were undetectable in 10 tumors and the other 2 showed very low ER levels. Tamoxifen treatment markedly reduced PgR levels, but specific progesterone receptor binding remained detectable in 9 out of 12 tumors examined. The mean PgR level before

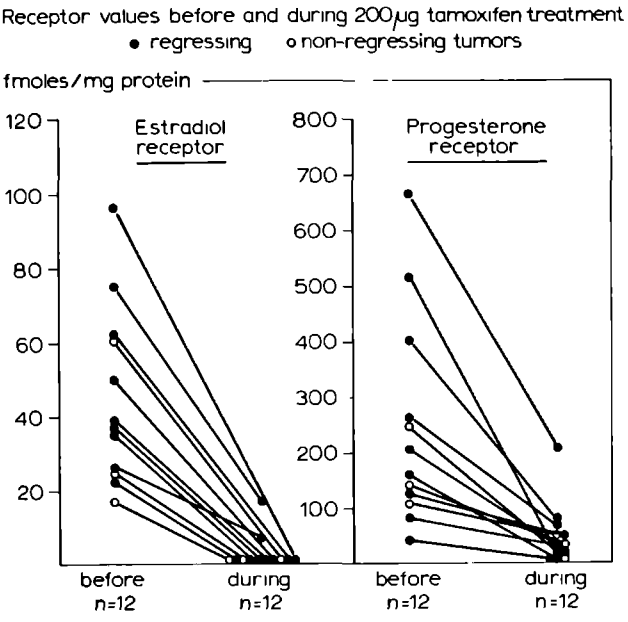


Fig. 4.3 Effect of 200 µg tamoxifen treatment on estradiol and progesterone receptor levels of regressing (•) and non-regressing (O) DMBA-induced rat mammary tumors.

treatment was 248 ± 55 and declined to 47 ± 17 fmoles/mg protein (80% reduction) after 3 weeks of 200 µg tamoxifen/day. Attention is drawn to the fact that ER and PgR levels declined in regressing as well as in non-regressing tumors.

4.5 *Estradiol and progesterone receptors after tamoxifen withdrawal in autonomous tumors*

Within 3-4 months after tamoxifen administration was started new tumors developed in every animal. Furthermore about 25% of the tumors that had regressed as a result of tamoxifen administration exhibited spontaneous tumor regrowth. After tamoxifen withdrawal these growing tumors did not respond to ovariectomy. Apparently tumor growth had become autonomous during tamoxifen.

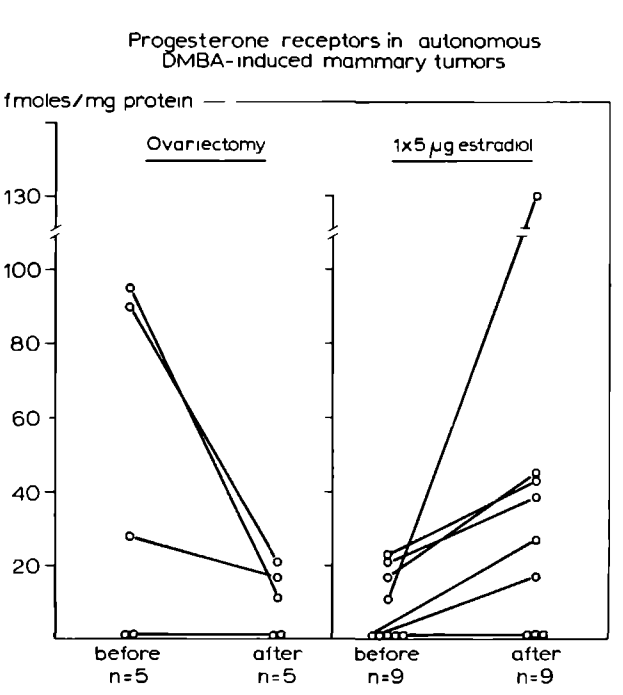


Fig. 4.4 Effect of ovariectomy and of one subsequent estradiol administration on progesterone receptor levels of autonomous DMBA-induced rat mammary tumors. Tumors that grew during 200 µg tamoxifen treatment and after tamoxifen withdrawal did not respond to ovariectomy were designated as autonomous.

Biopsies of these autonomous tumors have been taken on day 14 ± 0.7 (mean ± S.E., range 10-21 days) after tamoxifen withdrawal. In 12 out of 19 tumors analysed detectable amounts of PgR were present. The levels ranged from 0 to 170 fmoles/mg protein (39 ± 11, mean ± S.E.). Despite the fact that some tumors contained considerable PgR levels, ER levels were very low (<15 fmoles/mg protein) or undetectable (13/19) in all tumors (n=19) after tamoxifen withdrawal.

In these autonomous tumors it was investigated whether the synthesis of

PgR, as in normal target tissues and in ovarian-dependent DMBA-tumors, is under estrogen control. Therefore the effect of ovariectomy and of subsequent estradiol administration on PgR levels was studied. About 3 weeks after tamoxifen withdrawal the animals were bilaterally ovariectomized. As mentioned before these growing tumors did not respond to ovariectomy and were therefore designated as autonomous. Biopsies of these autonomous tumors were taken 11-22 days after ovariectomy. At the same day the animals received one injection of 5 µg estradiol as the benzoate (s.c.). Forty hours after this injection the animals were sacrificed and the tumors were excised for receptor analysis. The PgR levels of autonomous tumors measured before and after ovariectomy and after subsequent estradiol administration are collected in Fig. 4.4. It can be seen that ovariectomy decreased PgR levels in those 3 tumors, that contained detectable amounts of PgR before ovariectomy. A single injection of 5 µg estradiol as the benzoate increased the concentration of PgR binding sites in 6 out of 9 tumors studied. The remaining 3 tumors were PgR-negative before and after estradiol administration.

4.6 Discussion

Antiestrogens may elicit tumor regression of established DMBA-induced rat mammary tumors. In the present study 20% of the tumors regressed during daily administration of 50 µg of tamoxifen. In good agreement with several other reports 200 µg of tamoxifen caused tumor regression of 70% of the tumors (Jordan and Koerner, 1976; Nicholson and Golder, 1975). The regression of tumors to tamoxifen administration appeared to be related with the quantitative estradiol receptor binding capacity. Tumors, that regressed during the administration of 200 µg tamoxifen had significantly higher ER levels than non-regressing tumors. However there was no absolute division between both groups of tumors on the basis of ER levels, because the lower levels of regressing tumors and the higher levels of non-regressing tumors showed a considerable overlap. These observations are in accordance with recent reports of Jordan and Jaspan (1976) and Nicholson et al. (1977), who both observed that tumors which remained static or continued to grow during tamoxifen administration had very low ER values, whereas regressing tumors had higher ER values.

As appears from the results reported here, progesterone receptors were detectable in all regressing and in 5 out of 9 non-regressing tumors.

This indicates that there is no absolute relation between the presence or absence of PgR and the endocrine responsiveness of these tumors. Interestingly total absence of detectable PgR occurred only in tumors that failed to regress during tamoxifen. A tighter relation of tumor response to tamoxifen administration was obtained when both receptor levels were taken into account. This observation indicates that measurement of PgR along with ER provides additional although not definitive information with regard to responsiveness of DMBA-induced mammary tumors to tamoxifen treatment.

After the administration of 200 µg tamoxifen/day for a period of 3 weeks ER levels in the cytoplasmic fraction became very low or undetectable. Recently Tsai and Katzenellenbogen (1977) reported that in DMBA-tumor cells during treatment with the antiestrogen U-23, 469 (Upjohn Company) over 90% of ER was localised in the nucleus with concomitant low levels of ER in the cytoplasmic fraction. These investigators suggested that this situation may render the tumor cells insensitive to the animal's own endogenous estrogen and, hence, unable to grow.

During tamoxifen administration PgR levels markedly decreased in both the regressing and non-regressing tumors. During this endocrine intervention the effects on tumor growth and on PgR synthesis appearently were not related. Tsai and Katzenellenbogen (1977) recently analysed the effect of a new antiestrogen on PgR activity in DMBA-tumors, that regressed during this treatment. In contrast to our observation these investigators observed only a slight decrease in PgR levels, a difference that may be caused by divergent pathways in the mechanism of action of the antiestrogens used. Interestingly these investigators also emphasized dissociation between effects on growth and on PgR synthesis.

From our study it appeared that during long term tamoxifen treatment new growing tumors developed, whereas regressed tumors showed a spontaneous regrowth. After tamoxifen withdrawal these growing tumors did not respond to ovariectomy, suggesting that tumor growth had become autonomous. ER levels were very low or undetectable in these autonomous tumors after tamoxifen withdrawal. It is unknown, whether these very low ER levels are due to the previous tamoxifen treatment, resulting in a prolonged depletion of estradiol receptors in the cytoplasm, or are related to the fact that these tumors are autonomous. In most of these autonomous tumors PgR not only was detectable, but was still inducible by estradiol as is indicated by the decreased PgR levels after ovariectomy and the increased PgR levels after E_2 treatment.

CHAPTER 5

ESTRADIOL AND PROGESTERONE RECEPTORS IN PRIMARY AND METASTATIC HUMAN BREAST CANCER.

5.1 Introduction

In normal target tissues the concentrations of estradiol and progesterone receptors are regulated by steroid hormones (Bayard et al., 1978). There is ample evidence that also in DMBA-induced rat mammary tumors steroid hormones exert acute control over the production of ER and PgR. In DMBA-tumors estradiol receptors are stimulated by estradiol and prolactin, whereas progesterone inhibits ER synthesis (Sasaki and Leung, 1975; Leung and Sasaki, 1975; Kelly et al., 1978). On the other hand PgR synthesis in these tumors is induced by estradiol (see chapter 3).
The observation that tumors from premenopausal patients have lower ER levels than tumors from postmenopausal patients suggests that receptor levels in human breast tumors may be modulated by circulating steroid hormones (McGuire, Carbone and Vollmer, 1975).

In this chapter the distributions of ER and PgR in primary and metastatic human breast cancers are presented. The data were evaluated with special emphasis on the menopausal status and the use of contraceptive steroids. To our knowledge no studies have been reported regarding the influence of contraceptive steroids on ER and PgR levels in human breast cancer.

Recently it has been questioned whether receptor assays performed in the primary tumor at time of mastectomy can be used to guide endocrine therapy at time of recurrence (Jensen et al., 1975). In other words, is there a relation between receptor activity in the primary tumor and in metastatic lesions at time of recurrence? This would be of clinical importance because many patients have metastatic lesions, that are inaccessible for excision. To investigate this attractive possibility sequential receptor assays were performed in the primary breast tumor and metastases of the same patient. Furthermore multiple sequential biopsies of metastases of the same patient were analysed for the presence of ER and PgR in order to study the evolution of receptor activity during the course of this disease (Singhakowinta et al., 1976, Kiang et al., 1977).

There is only very limited information available in the literature about the effect of endocrine therapy on ER and PgR levels of human breast tumors (Walt et al., 1976). Moreover there is no conclusive information as to how much time is required before biopsies can be analysed for receptor activity after discontinuation of estrogen (ethynylestradiol) or antiestrogen (tamoxifen) treatment. Therefore sequential biopsies of metastases of the same patient have been analysed for receptor activity before, during and after tamoxifen or ethynylestradiol treatment. In addition tumors have been studied before and after ovariectomy.

5.2 Estradiol and Progesterone receptors in Primary and Metastatic Breast tumors

Biopsies of 297 primary and 91 metastatic breast tumors have been analysed for the presence of estradiol receptors (ER) and progesterone receptors (PgR). The incidence of both receptors in primary and metastatic breast tumors is presented in Table 5.1. Biopsies of metastases obtained within six weeks after tamoxifen withdrawal were excluded.

Table 5.1 Incidence of ER and PgR in human breast cancer.

	Total	Primary	Metastatic
ER-positive	71% (277/388)	74% (219/297)[a]	64% (58/91)[a]
PgR-positive	51% (199/388)	55% (165/297)[b]	37% (34/91)[b]

[a] $\chi^2 = 3.41$; DF = 1 ; $0.1>p>0.05$

[b] $\chi^2 = 9.23$; DF = 1 ; $p<0.01$

Of all tumors (n=388) analysed 71% were ER-positive and in 51% of the tumors PgR could be detected. The frequency of PgR-positive tumors was significantly higher ($p<0.01$) in primary (55%) than in metastatic tumors (37%). Primary breast tumors were also more likely to be ER-positive than metastases, the difference being close to statistical significance ($0.1>p>0.05$). The distribution of PgR-positive tumors, in relation to the presence or absence

of ER, can be seen in Table 5.2.

Table 5.2 Distribution of PgR-positive human breast tumors.

	Total	Primary	Metastatic
ER+ PgR+	68% (189/277)	72% (157/219)[a]	55% (32/58)[a]
ER- PgR+	9% (10/111)	10% (8/78)	6% (2/33)

[a] $\chi^2 = 5.77$; DF = 1 ; $p<0.02$

It appeared that the presence of PgR was essentially restricted to ER-positive tumors. In the group of ER-positive tumors 68% were also PgR-positive whereas only 9% of the ER-negative tumors were PgR-positive. The frequency of ER+ PgR+ tumors was significantly ($p<0.02$) higher in primary (72%) as compared to metastatic breast tumors (55%). In these series of patients, 10 (2.6%) showed PgR, whereas no ER could be detected. Most (7/10) of these tumors had very low PgR levels (<20 fmoles/mg protein). Six out of these 10 patients appeared to be premenopausal.

5.3 Estradiol and Progesterone Receptors in Primary Breast Tumors

5.3.1 Influence of Menopausal status on steroid hormone receptors

The influence of the menopausal status on the receptor distributions and the receptor levels was examined. A woman was considered as postmenopausal when menses had ceased at least one year prior to biopsy. The receptor distributions of these groups of patients is presented in Table 5.3. It appeared that postmenopausal patients were more likely ($\chi^2 = 3.57$; $0.1>p>0.05$) to have ER-positive tumors (78%) than premenopausal women (67%). The frequency of PgR-positive tumors however was not different for these two groups of women.

Table 5.3 ER and PgR distributions in primary breast tumors of pre- and postmenopausal patients.

	number of patients analysed	ER+ PgR+	ER+ PgR-	ER- PgR+	ER- PgR-
premenopausal	76	57%	10%[a]	7%	26%
postmenopausal	167	53%	25%[a]	2%	20%

[a] $\chi^2 = 6.83$; DF = 1 ; $p<0.01$

The difference in ER distribution between pre- and postmenopausal patients was due to ER+ PgR- tumors being significantly ($p<0.01$) more frequent in postmenopausal patients (25%) as compared to premenopausal patients (10%). The mean receptor levels of the receptor-positive tumors of these two groups of women are collected in Table 5.4.

Table 5.4 ER and PgR levels in primary breast tumors of pre- and postmenopausal women (ER- PgR- tumors excluded).

Patients	Estradiol Receptor mean ± S.E.	range[a]	Progesterone Receptor mean ± S.E.	range[a]
premenopausal	62 ± 7 (51)[b]	9-220	193 ± 29 (48)[c]	8-1250
postmenopausal	202 ± 19★(131)	20-1400	190 ± 28 (92)	8-1230

[a] all values are expressed as fmoles/mg protein

[b] the number of estradiol receptor-positive tumors is indicated in parentheses

[c] the number of progesterone receptor-positive tumors is indicated in parentheses

★ $p<0.01$ (pre-vs. postmenopausal women, Wilcoxon rank test).

The mean receptor level of the estradiol receptor-positive tumors of premenopausal patients was significantly lower than the corresponding mean receptor level of postmenopausal patients. No such difference was observed for the mean level of PgR.

5.3.2 Influence of Age on Steroid hormone receptors

In Figs. 5.1 and 5.2 the ER and PgR levels of primary breast tumors are plotted against the age of the patient.

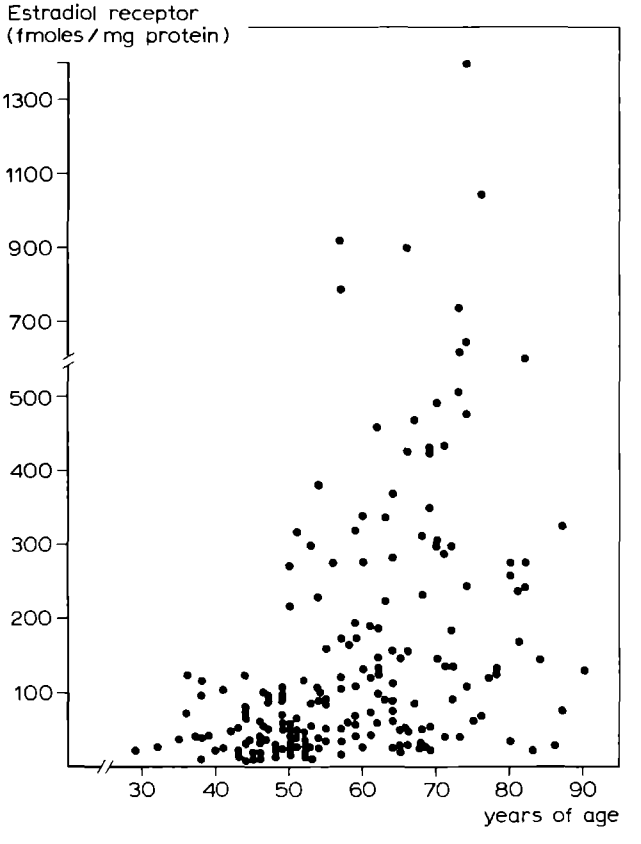


Fig. 5.1 ER levels in primary breast tumors plotted against the age of the patient.

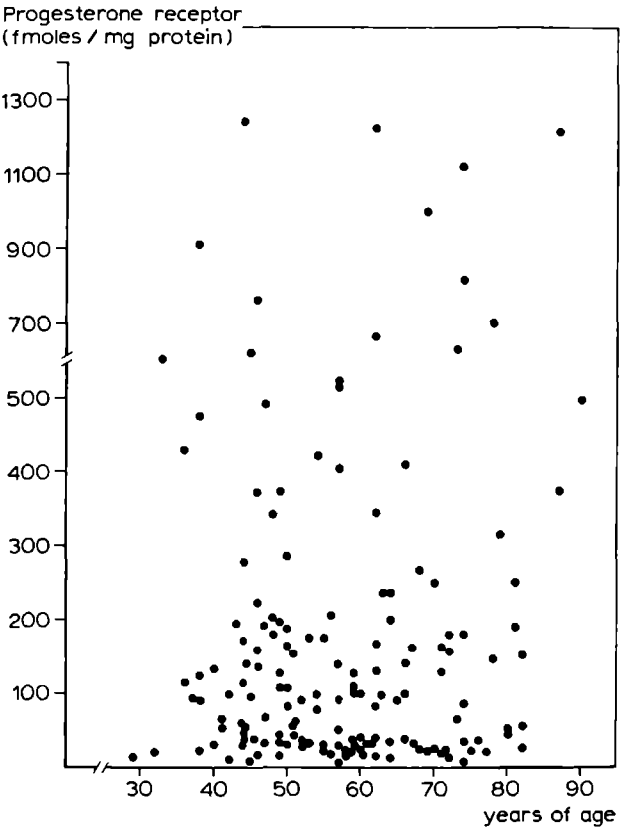


Fig. 5.2 PgR levels in primary breast tumors plotted against the age of the patient.

It is noteworthy that frequently ER levels of patients older than 50 years of age (predominantly postmenopausal) were higher than the upper level obtained in younger patients (predominantly premenopausal). No such difference was observed for PgR values. As Table 5.5 shows the frequency of ER-positive tumors increased with age. The difference between the percentage of ER-positive tumors in the group of women younger than 40 and older than 60 was close to statistical significance ($\chi^2 = 3.51$; DF = 1 ; $0.1 > p > 0.05$). There appeared to be no consistent influence of the age of the patient on the incidence of PgR-positive primary breast tumors.

Table 5.5 ER and PgR distribution in primary breast tumors: Influence of age.

age (years)	number of patients analysed	ER-positive (%)	PgR-positive (%)
<40	23	61	52
40-50	59	69	66
50-59	77	71	49
>60	115	79	57

5.3.3 Influence of contraceptive steroids on steroid hormone receptors

Some of the premenopausal patients used contraceptive steroids (Lyndiol^{R}, Stediril d^{R}, Neogynon^{R}, Microgynon^{R} and Orthonovum^{R}) just prior to tumor biopsy excision. Receptor distributions and levels were compared in two groups of premenopausal women; contraceptive steroid users and non-users. The ER levels of the non-users ranged from 0 to 120 fmoles/mg protein. Interestingly 6 out of 23 users with ER-positive tumors showed values above this level (120-220 fmoles/mg protein). The range of PgR levels was similar for both types of patients (0 to about 1000 fmoles/mg protein). No statistically significant differences in the frequency of ER-positive and PgR-positive tumors between these two groups of patients were observed (Table 5.6).

5.3.4 Correlation between estradiol and progesterone receptor binding sites in primary breast tumors

Estradiol receptor levels have been plotted against the corresponding progesterone receptor levels separately for pre- (Fig. 5.3) and postmenopausal patients (Fig. 5.4). No correlation was observed between the levels of ER-positive and PgR-positive tumors in either category of patients.

Table 5.6 ER and PgR distribution in primary breast tumors of premenopausal women in relation to use of contraceptive steroids.

	ER-positive	PgR-positive
patients using contraceptive steroids	74% (23/31)[a]	72% (13/18)[b]
patients not using contraceptive steroids	61% (34/56)[a]	61% (34/56)[b]

[a] $\chi^2 = 1.06$; DF = 1 ; $p>0.1$

[b] $\chi^2 = 0.36$; DF = 1 ; $p>0.5$

When both receptors were present, premenopausal women generally (74%) had a PgR/ER ratio above 1, whereas 61% of the postmenopausal women had a ratio below 1 ($\chi^2 = 13.74$; DF = 1 ; $p<0.001$). Although no correlation between the quantitative ER and PgR values was observed, there was a positive relation between the incidence of PgR-positive tumors and the concentration of estradiol receptor binding sites (Table 5.7).

Table 5.7 Comparison of estradiol receptor binding sites and the incidence of PgR-positive primary breast tumors.

ER (fmoles/mg protein)	number of patients analysed	PgR-positive
0	78	10%
<30	41	54%
31-50	29	66%
51-99	51	73%
>100	98	82%

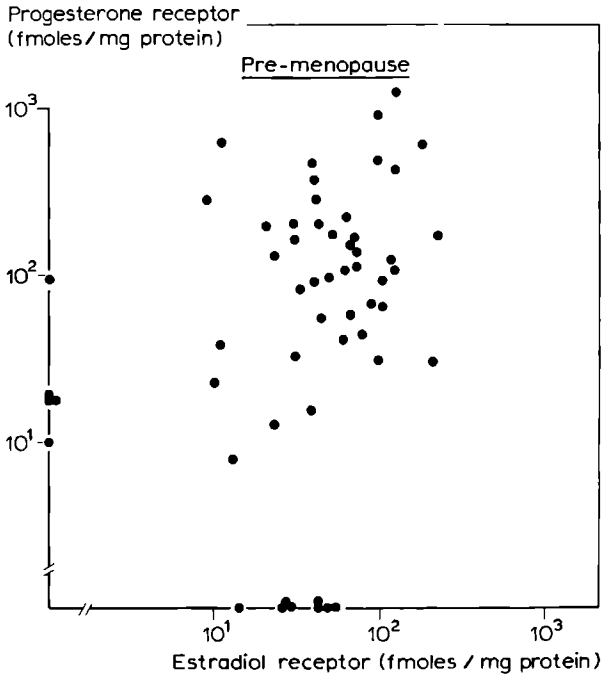


Fig. 5.3
Correlation between estradiol and progesterone receptor levels in primary breast tumors from premenopausal patients (n=76). Twenty breast tumors were regarded as ER- PgR-.

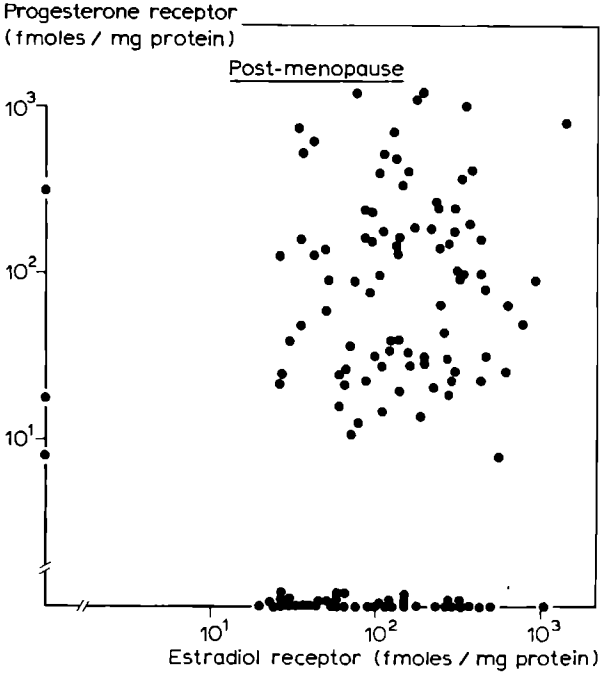


Fig. 5.4
Correlation between estradiol and progesterone receptor levels in primary breast tumors from postmenopausal patients (n=167). Thirty-three breast tumors were regarded as ER- PgR-.

5.4 Estradiol and progesterone receptors in normal tissues and in benign mammary tumors

Several biopsy specimens have been analysed for the presence of estradiol and progesterone receptors, which on histological examination appeared to be nonmalignant (normal breast tissue, skin biopsies and lymph nodes). None of these specimens had measurable ER (n=22) or PgR (n=13). Thirteen benign mammary tumors, mainly fibroadenomas, have been studies for the presence of ER and all were classified as ER-negative. Uptill now progesterone receptors have been analysed in only 3 benign mammary tumors and all 3 specimen appeared to be PgR-negative.

5.5 Estradiol and Progesterone Receptors in different tumor specimens of the same patient

5.5.1 Estradiol and progesterone receptors in two simultaneously excised biopsies

From 8 patients tumor biopsies were simultaneously excised from different tumor sites (Table 5.8). The tumors from 7 patients were classified identical, either both as ER-positive (n=3) or both as ER-negative (n=4). From 1 patient two osteolytic bone metastases have been analysed. One specimen revealed an ER value of 50 fmoles/mg protein, whereas in the other no ER could be detected. It is noteworthy that the histologic examination demonstrated that the tumor cellularity of these lesions was low. The tumor biopsies of 3 patients have also been analysed for the presence of PgR. In these 3 patients the classification of both metastases either as PgR-positive (n=1; PgR: 8 and 30 fmoles/mg protein, patient 3) or as PgR-negative (n=2, Patients 4 and 8) was the same.

Table 5.8 ER in multiple simultaneously taken tumor biopsies from the same patient.

patient	Anatomic sites	ER (fmoles/mg protein)	Anatomic site	ER (fmoles/mg protein)
1	lymph node	240	lymph node	70
2	breast	50	breast	20
3	breast	10	breast	15
4	breast	0	breast	0
5	lymph node	0	lymph node	0
6	skin	0	skin	0
7	ovary	0	ovary	0
8	bone	50	bone	0

5.5.2 Estradiol Receptors in two sequential biopsies of the primary breast tumor from the same patient

From 4 patients two sequential primary breast tumor biopsies have been analysed for the presence of ER. Two patients had an inoperable primary tumor and the other two showed recurrence of the primary breast tumor. Two patients had on both occasions ER-positive tumors and two patients had ER-negative tumors. The ER values of these two patients were 30 and 115, and 30 and 25 fmoles/mg protein respectively.

5.5.3 Sequential Estradiol Receptor assays in the primary breast tumor and metastases of the same patient

During the course of this investigation there was opportunity with 17 patients to obtain a tumor specimen from the primary tumor and at various time intervals also from a metastatic lesion. The ER values of these patients are collected in Fig. 5.5. It appeared that ER-positive primary tumors generally give rise to ER-positive metastases (5 out of 6 patients), whereas ER-negative primary tumors generally give rise to ER-negative metastases (10 out of 11 patients).

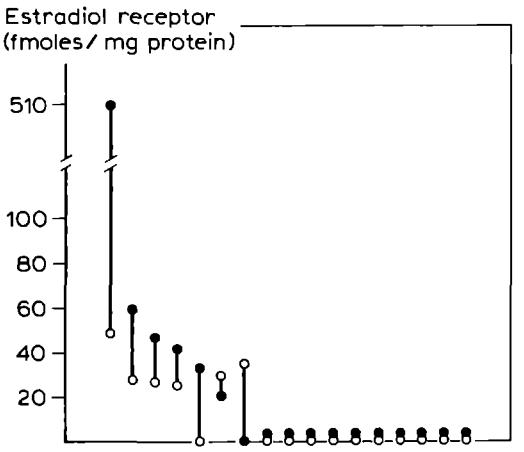


Fig. 5.5
Comparison of ER levels in sequential biopsies of the primary breast tumor (●) and metastases (O) of the same patient (n=17).

Only in 2 out of these 17 patients (12.0%) the receptor classification of the primary tumor and of the metastasis at time of recurrence was not in agreement. One patient had an ER value of 35 fmoles/mg protein in the primary breast tumor, 29 months later a lymph node metastasis appeared to be ER-negative, however with a PgR value of 55. This patient was still menstruating and the biopsy specimen was excised during the follicular phase of the cycle. The other patient had an ER- PgR- primary tumor. Six months later a metastasis revealed an ER value of 35 and a PgR value of 110 fmoles/mg protein. Five out of 6 patients with ER-positive primary tumors showed lower ER values in the later appearing metastases.

5.5.4 Multiple sequential receptor assays in metastases of the same patient

Several patients were followed for extensive periods and with 23 patients there was opportunity to obtain different metastatic biopsy specimens during the course of their disease. Most patients received some form of treatment (radio-, chemo- or endocrine therapy) in the interval between the tumor excisions. The ER values of these patients are collected in Fig. 5.6.

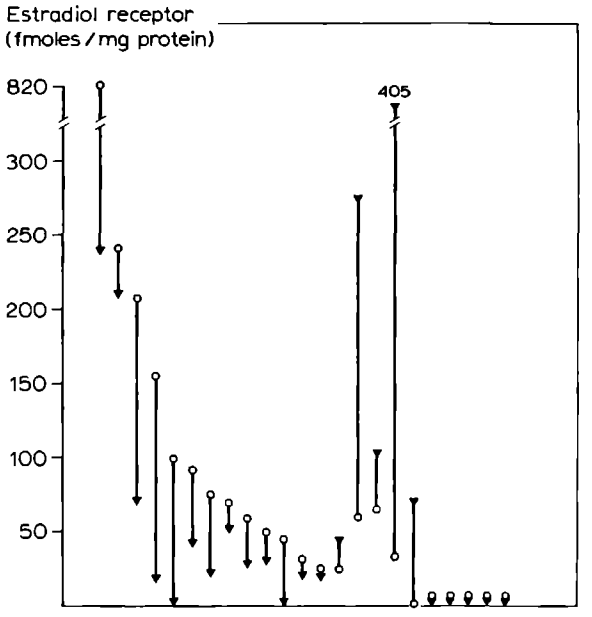


Fig. 5.6 Sequential estradiol receptor assays in metastases of the same patient (n=23).
o *first biopsy ;* ▼ *second biopsy*

Metastases of the same patients excised at different times during the course of their disease generally were both ER-positive (15 out of 17 patients = 88%) or both ER-negative (5 out of 6 patients = 83%). Two patients showed ER-positive skin metastases and revealed ER-negative metastatic tumors 3 weeks after termination of ethynylestradiol administration and 6 weeks after tamoxifen withdrawal respectively. One patient had an ER-negative skin nodule and subsequently received 5-fluoro uracil. Ten months later another skin nodule revealed an ER value of 70 fmoles/mg protein. From 13 patients the ER value of the second biopsy was lower and from 5 patients higher compared to the ER value of the formerly excised biopsy specimens. In 4 patients, which on two former occasions had ER positive metastatic lesions, a third metastasis was available for study.

Three of these patients again revealed ER-positive tumors, whereas in the fourth patient no ER could be detected.

From 8 patients different tumor biopsies have also been studied for the presence of PgR. The results are collected in Table 5.9. Although the number of observations is small, it appeared that in 4 out of 5 cases progesterone receptor became undetectable during the course of their disease.

Table 5.9 Comparison of PgR values in metastatic tumor biopsies from the same patient excised at different times.

Patient	first metastasis	second metastasis	third metastasis
1	720	0	0
2	580	0	
3	25	30	
4	20	0	
5	15	20	0
6	0	0	
7	0	0	
8	0	0	

The data are expressed as fmoles/mg protein.

5.6 Steroid hormone receptor levels before, during and after endocrine treatment in biopsies of the same patient

5.6.1 Estradiol receptor levels before, during and after tamoxifen administration

The ER levels measured before, during and after tamoxifen administration in sequential biopsies of the same patients are collected in Fig. 5.7. The ER levels before treatment ranged between 20 and 480 fmoles/mg protein. During tamoxifen administration no cytoplasmic ER binding could be detected (n=8).

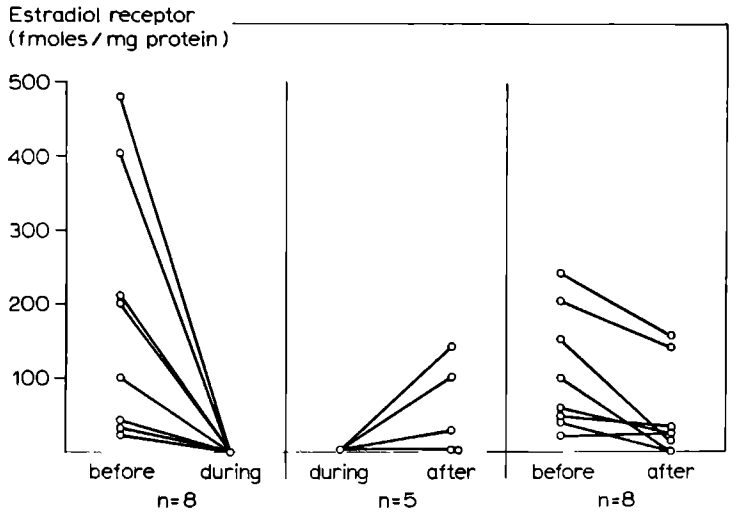


Fig. 5.7 ER levels before, during and after tamoxifen (40 mg daily) administration in sequential biopsies of the same patients.

In 3 out of 5 patients ER binding increased from 0 during tamoxifen treatment to values, that ranged between 30 and 145 fmoles/mg protein after drug withdrawal. In 8 patients ER was measured before as well as after tamoxifen treatment. After tamoxifen withdrawal the ER values appeared to be considerably lower compared to the corresponding values measured before this treatment was started. Two patients with ER-positive metastases before therapy revealed ER-negative tumors 6 and 8 weeks after termination of this therapy. The interval between drug withdrawal and tumor excision varied between 2 weeks and 3 months in the other 6 patients with ER-positive tumors after tamoxifen treatment.

5.6.2 Estradiol and progesterone receptor levels before, during and after ethynylestradiol administration

The results of these observations are summarized in Table 5.10. No cytoplasmic estradiol receptor binding could be detected in biopsies of 3 patients during ethynylestradiol administration (3000 µg/daily).

During this treatment progesterone receptors however, in contrast to estradiol receptors, were clearly present in all 3 patients studied. After drug withdrawal 6 out of 7 patients again had ER-positive metastases. In one patient ER binding sites could not be detected in two metastatic lesions excised 3 weeks and 4 months after cessation of this treatment. In the other patients with ER-positive tumors, biopsies were obtained 4 weeks or later after ethynylestradiol withdrawal. In Table 5.10 it can be seen that after ethynylestradiol withdrawal the ER values of 3 patients were lower and of 3 patients were higher compared to the corresponding values measured before therapy.

Table 5.10 Steroid hormone receptor levels before, during and after ethynylestradiol administration.

before	during	
ER	ER	PgR
210[a]	0	-
60	0	100
45	0	720
-	-	460

before	after	Interval[b]
ER	ER	
820	240	5 weeks
210	140	2 years
65	100	4 weeks
45	0	3 weeks
35	410	8 weeks
30	120	4 weeks
25	30	2 years

[a] All values are expressed as fmoles/mg protein

[b] Time interval between ethynylestradiol withdrawal and second biopsy.

5.6.3 Estradiol and progesterone receptor levels before and after ovariectomy

From 5 patients specimens have been analysed before as well as after ovariectomy (Table 5.11). All patients showed ER-positive tumors before as well as after ovariectomy. However it may be noted that the quantitative ER levels in 4 out of 5 patients were lower after ovariectomy. In 1 ovariectomized woman an ER value of 275 fmoles/mg protein was obtained. This value was higher than the highest value observed in the group of premenopausal patients studied (Table 5.4). From several other patients ER values have only been measured after ovariectomy. Also in this group of patients occasionally very high ER values were observed up to 490 fmoles/mg protein. Therefore it seems that at least in some patients ER values increase when they change of menopausal status, because of the removal of their ovaries. In one out of three patients the PgR assay was negative 3 weeks after the performed ovariectomy.

Table 5.11 ER and PgR levels before and after ovariectomy.

before		After		Interval[b]
ER	PgR	ER	PgR	
90[a]	15	40	20	8 weeks
70	580	50	0	3 weeks
60	25	275	30	21 months
75	-	25	-	16 months
60	-	30	-	36 months

[a]All receptor values are expressed as fmoles/mg protein

[b]Time interval between ovariectomy and second biopsy.

After the suggestion of McGuire et al. (1975) that PgR could be a sensitive marker for prediction of response to endocrine therapy, various laboratories have developed an assay for the measurement of PgR in biopsies of human breast tumors. Today at least 15 laboratories have published data regarding ER and PgR in human breast tumors★. In these studies the percentage of receptor-positive tumors varied between 53 and 86% for ER and between 27 and 57% for PgR. In only 4 out of these 15 studies information was given about the origin of the tumor tissue, whether the biopsy was taken from the primary tumor or from a metastatic lesion, and about the menopausal status. In the present study 71% of all tumors analysed were ER-positive and in 51% of the tumors PgR could be detected. This wide variation of receptor-positive tumors may be due to:

1. collection and storage conditions especially regarding the thermolability of PgR binding sites (chapter 2).
2. the procedures used for the determination of receptor activity e.g. buffer composition, the type of radioactive ligand used and the method used for separation of free and bound steroid.
3. the criteria used for classification tumors either as receptor-positive or as receptor-negative e.g. several investigators have arbitrarily defined cut off levels of 20-25 fmoles/mg protein for PgR and every tumor showing a lower value is classified as receptor-negative.
4. a different incidence of receptor-positive breast tumors in patients living in different parts of the world e.g. Matsumoto and Sugano (1978) reported that tumors from American patients more frequently contained ER than tumors from Japanese patients.

Despite this wide variation in the frequency of PgR-positive tumors all investigators agreed that the presence of PgR was essentially restricted to ER-positive tumors. In these studies 2-12% of all tumors analysed contained PgR without ER. In this laboratory the frequency was 2.6%.

★ (Saez et al., 1978; Matsumoto and Sugano, 1978; Young et al., 1978; Rao and Meyer, 1977; May-Levin et al., 1977; Daehnfeldt et al., 1977; Tobin et al., 1977; Pichon and Milgrom, 1977; Leclercq et al., 1977; Horwitz and McGuire, 1977; DeSombre and Jensen, 1977; Wittliff et al., 1978; Daxenbichler et al., 1977; Caffier and Brandau, 1978; Barnes et al., 1977).

Interestingly several investigators (Pichon and Milgrom, 1977; Saez et al., 1978; Horwitz and McGuire, 1977), as in the present study (Table 5.7), observed that the incidence of PgR-positive tumors was positively related with increasing ER levels. Tumors with high ER levels were more likely to be PgR-positive than tumors with low ER levels. This finding suggests that in human breast cancer the progesterone receptor is under estrogen control.

In most studies primary breast tumors were slightly more often ER-positive than metastatic tumors, although the difference, as in the present study, was not always significant (Beex, 1979; McGuire, Carbone and Vollmer, 1975; Table 5.1). Several investigators have performed sequential ER assays in the primary breast tumor and metastases from the same patient. From these reports (Jensen et al., 1975; Rosen et al., 1977; Matsumoto and Sugano, 1978; Maass et al., 1975), as from the present study (Fig. 5.5), it appeared that an ER-positive primary tumor generally gives rise to an ER-positive metastasis, whereas an ER-negative primary tumor gives rise to an ER-negative metastasis. Therefore it can be expected that ER assays performed in the primary tumor can be used to guide endocrine treatment or other treatments, when a patient subsequently develops metastatic disease. Recently DeSombre and Jensen (1977) reported that the ER value of the primary breast tumor predicted response to endocrine treatment much later, at time of recurrence.

In accordance with recent reports of Leclercq et al. (1977) and Horwitz and McGuire (1977) it was observed that ER-positive primary breast tumors were significantly more frequent PgR-positive than metastatic breast tumors. In addition it appeared from the present study that in 4 out of 5 patients PgR became undetectable during the metastatic period of their disease (Table 5.9). These observations suggest that the ability to synthesize PgR is lost in dedifferentiating metastasizing tumors.

Various investigators have studied the influence of the menopausal status on steroid hormone receptors. It has consistently been observed that tumors from premenopausal patients contain lower ER values than tumors from postmenopausal patients (Wittliff, 1974; McGuire, Carbone and Vollmer, 1975; Matsumoto and Sugano, 1978; Table 5.4). These lower ER levels may be related to the higher levels of circulating endogenous estrogens in these patients, which could occupy free cytoplasmic receptor sites and thus make them unavailable with the assays used.

Therefore several investigators have studied, whether high plasma estrogen levels or high concentrations of endogenous estrogens in tumor cytosols were related to low or negative ER measurements (Maass et al., 1975; Fishman et al., 1977; Theve et al., 1978). From these studies it appeared that no relationship exists between ER binding and endogenous estrogen levels, although in a few patients with very high plasma estradiol levels no ER binding was detectable. In fact estradiol levels were higher in cytosols from ER-positive tumors, compared to cytosols from ER-negative tumors (Fishman et al., 1977; Maynard et al., 1977). Moreover Sakai and Saez (1976) showed that the underestimation of cytoplasmic receptor values, because of occupation of free receptor sites, is too low to explain the observed difference between pre- and postmenopausal patients. However these studies do not exclude the possibility that estradiol receptor complexes have translocated to the cell nucleus and therefore were no longer detectable in the cytoplasmic fraction. Bayard et al. (1978) have reported that in human endometrium tissue both estradiol and progesterone total receptor concentrations (cytoplasmic + nuclear) were highest in the preovulatory phase and were significantly lower in the late secretory phase. The distributions of both receptors between the cytoplasmic and nuclear compartment was dependent on the circulating levels of estradiol and progesterone. About 50% of both receptors were present in the nucleus at the time that these steroid hormones were highest during the menstrual cycle. Recently Saez et al. (1978) showed that the mean cytoplasmic ER level in human breast tumors is also lower at the end of the cycle, but the difference was not significant. These investigators suggested that the lower ER values in premenopausal women are perhaps due to the fact that the cyclic progesterone increase limits estrogen stimulation of ER synthesis. This suggestion is in agreement with recent findings, which demonstrate that progesterone in vivo as well as in vitro can suppress estrogen receptor synthesis (Sasaki and Leung, 1975; Hsueh et al., 1975).

PgR levels, in contrast to ER levels, were not different in pre- and postmenopausal patients and the incidence of PgR-positive tumors was also similar in both groups of patients (May-Levin et al., 1977; Leclercq et al., 1977; Saez et al., 1978; McGuire, 1978; Tables 5.3 and 5.4). Remarkably May-Levin etal. (1977) and McGuire (1978), as in the present study, observed that tumors from postmenopausal patients were more often ER+ PgR- (23-30%) than tumors from premenopausal patients (8-12%).

Saez et al. (1978) found that no PgR was detectable in human breast tumors from 32 premenopausal patients, who had plasma progesterone levels above 100 ng/100 ml, whereas 15 out of 40 (37%) premenopausal patients with lower plasma levels were PgR-positive. It is worth mentioning that in the study of Saez et al. (1978) the incidence of ER+ PgR- tumors in premenopausal patients was 3 times higher (38%) than in the reports mentioned above (8-12%).

Several investigators have performed multiple simultaneous ER assays and without exception noticed that there are patients, who at the same time have ER-positive and ER-negative tumors (Rosen et al., 1977; Kiang and Kennedy, 1977; Singhakowinta et al., 1976). In this study 7 out of 8 tumors were classified identical. In most studies a similar frequency (85-90%) was found. In one report 8 out of 25 (33%) cases were ER-positive at one site and ER-negative at another (Webster et al., 1978). It has been suggested that this phenomenon may explain why some patients with ER-positive tumors failed to respond to endocrine treatment.

In the present study it appeared that multiple sequential biopsies from the same patient generally were both ER-positive or ER-negative. In 84% of 37 patients from whom at least two sequential biopsies - from the primary breast tumor and a metastasis or from different metastatic lesions - have been analysed for the presence of ER both tumors were classified identical (in 3 patients sequential biopsies were taken once from the primary tumor and twice from metastases). In a number of reports (Rosen et al., 1977; Leung et al., 1975; Matsumota and Sugano, 1975; Webster et al., 1970) differences in ER classification occurred in 11 to 28% of the cases or even higher than 50% (Engelsman, 1978). Although in these reports described in the literature occasionally a patient with an ER-negative lesion subsequently revealed an ER-positive lesion, the more frequent change was for a positive lesion to become negative. Because of the possibility that the receptor status of a patient has changed during the cause of her disease, receptor assays should be performed, whenever possible just prior to instituting treatment. Little information is available on quantitative ER levels in sequential biopsies. In this study ER levels in most patients declined as their disease progressed (Figs. 5.5 and 5.6). Walt et al. (1976) showed that in 14 out of 15 patients ER levels substantially declined in serial biopsies. In the latter study all patients received endocrine treatment and a few also chemotherapy during the interval between the receptor assays.

Two hypotheses exists to explain that tumors loose receptor activity and eventually become ER-negative. First, tumors are composed of dependent (ER-positive) and independent (ER-negative) cells. During endocrine therapy the growth of dependent cells is inhibited, allowing independent cells to become dominant (Sluyser, 1977). Secondly, during the course of this disease individual cells become more autonomous and loose the ability to produce ER (Singhakowinta et al., 1976).

In a few patients receptor assays were performed prior and during the administration of tamoxifen or ethynylestradiol. During both types of therapy ER became undetectable. These findings are in accordance with those of Carola et al. (1974), Engelsman (1978) and Singhakowinta et al. (1976), who also observed a complete loss of cytoplasmic ER activity during antiestrogen and estrogen therapy. It is clear that tissues of patients receiving estrogen or antiestrogen therapy are not suitable for receptor studies. During ethynylestradiol high PgR levels were observed in the tumors of the 3 patients that were studied (Table 5.10). One patient responded and the other two failed to respond to this therapy. This observation indicates that the effects of ethynylestradiol on tumor growth and on PgR synthesis were dissociated. There is no conclusive information in the literature as to how much time is required for a breast tumor to become ER-positive again after discontinuation of estrogen or antiestrogen therapy. From the data presented in this study it followed that 12 out of 15 patients showed ER-positive tumors 4 weeks or later after these treatments were stopped (5.6). The remaining 3 patients showed ER-negative tumors 3, 6 and 8 weeks after discontinuation of ethynylestradiol or tamoxifen therapy.

The collected results of 8 institutions on a total of 245 patients show that measurement of PgR along with ER enhances the ability to select patients for endocrine therapy (DeSombre and Jensen, 1977; McGuire, 1978). The response rate of patients, whose tumors contained both receptors was 74% (70/95), whereas the response rate of patients, whose tumors contained ER but no PgR was only 28% (21/74). Especially the last years the importance of quantitative receptor measurement has been recognized. Several investigators showed that a positive relation exists between the concentration of ER binding sites and the response to endocrine treatment (Jensen et al., 1975; Heuson et al., 1976; Engelsman, 1978; McGuire, 1978; Westerberg et al., 1978).

These data are the more impressive if one takes into account that the results were expressed in inadequate parameters such as fmoles/g wet tissue or as fmoles/mg protein. Since a positive correlation exists between the quantitative ER levels and the presence of PgR (Table 5.7) it is possible that quantitative ER levels or PgR measurements identify the same group of patients with the highest probability of obtaining a remission to endocrine therapy.

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Young, P.C.M., Einhorn, L.H., Ehrlich, C.E., Cleary, R.E. and Rohn, R.J. (1978): Proc. Am. Assoc. Cancer Res. 19, 204.

The scope of this investigation of steroid hormone receptors is closely connected with the clinical study described by Beex in his concomitant thesis. Both studies were designed to obtain further information about the relation between steroid hormone receptors and endocrine responsiveness of breast carcinoma.

A brief review of the vast literature on steroid hormone receptors and hormone dependency of tumor tissues and the scope of this thesis have been presented in Chapter 1.

Chapter 2 describes the method developed for the determination of estradiol and progesterone receptor activity. The multiple point dextran-coated charcoal assay allows construction of a Scatchard plot even when relatively small amounts of tissue (100 mg) are available for analysis. As the radioactive ligands, ^{3}H-estradiol was used for ER and ^{3}H-R5020 for PgR assay.

The criteria used to classify a tumor as receptor-positive were a plot with a linear portion and a calculated K_d value lower than 4 nM. It appeared that receptor values were underestimated when the protein content of the cytosol was lower than 2 mg per ml. Such cytosols may be falsely scored as receptor-negative. The lower limit of sensitivity was about 40 fmoles per ml cytosol for both the estradiol and progesterone receptor. In terms of fmoles per mg of cytosol protein, the lowest values actually measured were 5 and 8, respectively.

From studies regarding the possible interference of plasma proteins such as Sex Hormone Binding Globulin with the ER assay, it was concluded that such interference, if present, was slight and occurred only when tumors showed a relatively low receptor value. The virtual absence of interference from plasma proteins, especially SHBG, with the ER assay was confirmed by using ^{3}H-moxestrol, a ligand which does not bind to this protein. Experiments in which two different radioactive ligands (R5020 and Org 2058) were used for the assay of PgR strongly indicated that no serious interference from plasma proteins or glucocorticoid receptor binding sites occurred.

It is emphasized in the literature that there is a need for intra- and inter-laboratory quality control of receptor measurements.

In the present study, this problem was approached by preparing a lyophilised calf uterus powder which was found to be appropriate for such purpose.

Chapter 3 reports new experimental observations of the endocrine responsiveness of DMBA rat mammary tumors in relation to quantitative receptor values. Essentially all DMBA-tumors contained detectable amounts of ER. The ER levels of endocrine responsive tumors were significantly higher than those of unresponsive tumors; however, no absolute distinction could be made between both groups of tumors on the basis of ER levels.

It has been hypothesized by McGuire et al. (1975) that for prediction of endocrine responsiveness of breast tumors, the progesterone receptor - being an end product of estrogen action - would be a better marker than the estradiol receptor itself.

The results of this study do not support this hypothesis. Endocrine unresponsive DMBA-tumors can contain significant amounts of cytoplasmic progesterone receptor activity. Furthermore, it was shown that PgR in DMBA-tumors is under acute control of estradiol. Such control was not restricted to ovarian-dependent tumors, but also was observed in ovarian-independent tumors. These observations point to a dissociation between estradiol responsiveness and estradiol-induced PgR synthesis. Nevertheless, tumor regrowth occurred with estradiol administration in those tumors that had relatively high pre-ovariectomy ER and PgR levels.

In Chapter 4 it was reported that the regression of tumors upon administration of the antiestrogen tamoxifen appeared to be related with ER levels; regressing tumors had higher ER levels than non-regressing tumors. Again, however, there was no absolute division between the two groups of tumors. Progesterone receptors were detectable in all regressing and in about half of the non-regressing tumors. Total absence of detectable PgR occurred only in tamoxifen-unresponsive tumors. Thus, measurement of PgR along with ER provides additional although not definitive information with regard to this type of endocrine responsiveness.

Interestingly, during long term (2-3 months) tamoxifen treatment, regressed tumors showed spontaneous regrowth and new growing tumors developed. Because these tumors did not respond to ovariectomy after tamoxifen withdrawal, they can be regarded as autonomous. In these tumors, ER levels were very low or undetectable. Remarkably, PgR was not only detectable but still inducible by estradiol in most of these autonomous tumors.

This observation is another illustration that hormone independency can accompany intact estradiol-inducible PgR synthesis.

In Chapter 5, data are collected which are of relevance to the interpretation of receptor measurements in human mammary carcinoma. Although ER as well as PgR levels of primary and metastatic tumors showed a wide range of values, in none of the biopsies of benign tumors or normal breast tissue was receptor activity detectable. ER classifications of different tumor specimens taken from the same patient - whether simultaneously or sequentially, from primary tumor to metastasis or from metastasis to metastasis - were generally (in 49 of 56 occasions) in agreement. ER became undetectable during tamoxifen or ethynylestradiol treatment, which means that receptor analysis under such conditions is of little value. Several weeks may be required after discontinuation of such therapies before receptor studies are relevant. If all of the 388 tumors analysed are considered, 71% were ER-positive. Of these ER-positive tumors, 68% contained also PgR, wherewas in only 9% of the ER-negative tumors was PgR detectable. Actually, the percentage of PgR-positive tumors was positively correlated with the level of ER. These findings strongly indicate that in these mammary carcinomas, as in normal target tissues and in DMBA-induced rat mammary tumors, the synthesis of PgR depends on the action of estrogen. Furthermore, it appeared that the frequency of PgR-positive tumors was significantly higher among primary than among metastatic tumors. In addition, of the ER-positive primary tumors, 72% had detectable levels of PgR whereas only 55% of ER-positive metastatic tumors contained PgR. These findings indicate that endocrine control mechanisms are lost when the tumor cells become more dedifferentiated. In line with this assumption were the few observations that PgR became undetectable during the metastatic period of the disease.

From these observations and considerations, it emerges that it is likely that progesterone receptor measurements, along with the assay of ER, may improve the selection of endocrine responsive tumors. From a recent report (McGuire, 1978) in which the clinical correlation data of various investigators were collected, it appeared that, indeed, in the presence of both ER and PgR the chance of endocrine responsiveness was 74%. Objective response of patients in which ER was positive, irrespective of PgR was 53%.

In a small series of patients collected in our institution (Beex et al., unpublished observations) ER+ PgR+ tumors apparently showed more objective remissions with endocrine therapy than ER+ PgR- tumors. The hypothesis of McGuire et al. has thus received sufficient support from these studies to warrant continued research in this direction.

Het hier beschreven onderzoek is steeds nauw verbonden geweest met dat van internist Beex. Zijn klinische studies zijn verwerkt in een gelijktijdig verschenen proefschrift. Het gemeenschappelijke onderwerp is de relatie tussen steroid hormoon receptoren en hormoongevoeligheid van het mammacarcinoom.

De zeer omvangrijke literatuur die over dit onderwerp bestaat, is summier behandeld in hoofdstuk 1, waarin ook de doelstellingen van het onderzoek staan beschreven.

In hoofdstuk 2 volgt een beschrijving, alsmede een evaluatie, van de methodieken die werden ontwikkeld voor de bepaling van oestrogeenreceptoren (ER) en progesteronreceptoren (PgR). Voor de bepaling van ER en PgR werden ^{3}H-E_2 respectievelijk ^{3}H-R5020 gebruikt. Het bleek bij toepassing van deze methoden mogelijk om van slechts kleine hoeveelheden tumorweefsel (100 mg) uit te gaan terwijl toch elke afzonderlijke receptor bepaling, middels de "meerpuntsmeting" op specificiteit kon worden getoetst. De uit de "meerpuntsmeting" geconstrueerde Scatchard curve levert niet alleen informatie over de specificiteit maar ook over de onderste gevoeligheidsgrens van de bepaling. Dit laatste is van groot belang aangezien de beoordeling van een tumor als zijnde receptor-positief of receptor-negatief belangrijke konsekwenties heeft voor de behandeling van patienten met een gemetastaseerd mammacarcinoom. Blijkens het onderzoek is de gevoeligheid van de bepaling mede afhankelijk van de eiwitconcentratie van het tumorhomogenaat. Het verdient aanbeveling om bij de uitslag van de receptor analyse de eiwitconcentratie van het cytosol te vermelden. De gevoeligheid van de methode werd bij benadering vastgesteld op 40 fmol per ml cytosol. Uitgedrukt in fmol/mg eiwit waren de laagst gemeten receptor concentraties 5 voor ER en 8 voor PgR.

Uit de resultaten van studies, waarin de mogelijke interferentie van plasma eiwitten zoals het Sex Hormone Binding Globulin (SHBG) met de bepaling van ER werd bestudeerd, bleek dat deze interferentie, indien aanwezig, slechts gering was en alleen optrad in tumoren met een relatief lage receptor concentratie. De nagenoeg afwezige interferentie werd bevestigd d.m.v. studies, waarbij gebruik werd gemaakt van een ligand met slechts een zeer lage affiniteit voor SHBG (^{3}H-moxestrol).

Ook de eventueel storende invloed van plasma eiwitten en glucocorticoid receptoren op de meting van PgR werd geanalyseerd. Uit de resultaten van experimenten, waarbij twee verschillende liganden werden gebruikt (^{3}H-R5020 en ^{3}H-Org 2058), bleek niets van een dergelijke storing.

Het resultaat van een receptor analyse is niet alleen van de gekozen methodiek afhankelijk, maar eveneens van een groot aantal vaak nog onbekende factoren. Dientengevolge bestaat er behoefte aan een stabiel receptor preparaat dat in elke reeks van bepalingen kan worden meegenomen. Op deze wijze is een intra- en inter-"assay" variatiecoëfficiënt vast te stellen en bovendien kunnen laboratoria onderling hun resultaten vergelijken. Uit het beschreven onderzoek is gebleken dat door lyophilisatie van kalfsuterus weefsel een dergelijk preparaat kan worden bereid.

In hoofdstuk 3 staan studies beschreven naar de relatie tussen receptor concentraties en de hormoongevoeligheid van door dimethylbenzanthraceen bij ratten geinduceerde mammatumoren. In nagenoeg alle DMBA-tumoren kon ER worden aangetoond. In ovarium-afhankelijke tumoren bleek de concentratie van de oestradiolreceptor significant hoger te zijn dan in ovarium-onafhankelijke tumoren. Echter beide groepen van tumoren konden niet volledig van elkaar onderscheiden worden op basis van de ER concentraties.

McGuire en medewerkers (1975) hebben als hypothese gesteld dat hormoongevoeligheid van het mammacarcinoom mogelijkerwijs beter gedetecteerd zou kunnen worden door meting van de progesteron receptor, als zijnde een eindproduct van oestrogene activiteit.

De gegevens van dit hoofdstuk geven niet direct steun aan deze hypothese. Het bleek, dat ovarium-onafhankelijke tumoren aanzienlijke PgR concentraties kunnen bezitten. Bovendien werd aangetoond dat zowel in ovarium-afhankelijke als ook in ovarium-onafhankelijke DMBA-tumoren de PgR synthese onder directe invloed staat van oestradiol. Deze waarnemingen duiden op een ontkoppeling van oestradiol gereguleerde tumorgroei en oestradiol geinduceerde PgR synthese. Niettemin trad tumorhergroei door toediening van oestradiol slechts op in tumoren, die vóór de ovariectomie relatief hoge oestradiol en progesteron receptor concentraties vertoonden.

Uit de resultaten van hoofdstuk 4 blijkt dat er een relatie bestaat tussen tumor regressie, bewerkstelligd door toediening van het antioestrogeen tamoxifen en de hoogte van de oestradiol receptor concentratie. De ER concentratie was hoger in tumoren die regressie vertoonden dan in tumoren waarvan de tumorgroeisnelheid niet-meetbaar veranderde door de toediening

van tamoxifen. Ook in dit experiment bleek, dat beide groepen van tumoren niet volledig van elkaar onderscheiden konden worden, noch op basis van ER noch op basis van PgR activiteit. In alle tumoren die regressie vertoonden werd PgR activiteit aangetoond, evenals in ongeveer de helft van de tumoren waarbij geen teruggang van tumorweefsel optrad. Hieruit blijkt dat het meten van PgR naast ER weliswaar additionele, echter geen volledige, informatie geeft met betrekking tot dit type van hormoongevoeligheid.

Tijdens langdurige (2-3 maanden) tamoxifen toediening lieten tumoren, waarbij eerder een teruggang van tumorweefsel was opgetreden, spontane hergroei zien en ontwikkelden zich nieuwe tumoren. Aangezien deze tumoren niet reageerden op het verwijderen van de ovaria, nadat de tamoxifen toediening gestaakt was, werden deze beschouwd als zijnde autonoom. De ER concentraties in deze tumoren waren zeer laag tot niet-meetbaar. Opmerkelijk was dat de PgR activiteit, die in de meeste van deze autonome tumoren aantoonbaar was, nog steeds geinduceerd kon worden door oestradiol. Blijkbaar kan hormoononafhankelijkheid samengaan met het intact zijn van het mechanisme waardoor oestradiol PgR synthese stimuleert.

In hoofdstuk 5 zijn gegevens verzameld die van belang geacht kunnen worden voor de interpretatie van receptor metingen in humane mammacarcinomen. Alhoewel de ER en PgR concentraties in primair en metastatisch tumorweefsel een zeer grote spreiding lieten zien, bleek in geen van de geanalyseerde biopten van goedaardig tumorweefsel of normaal borstweefsel receptor activiteit aantoonbaar. De ER uitslag van verschillende tumorbiopten van eenzelfde patient - of de biopsie nu gelijktijdig of op verschillende tijdstippen plaatsvond (eerst in de primaire tumor en vervolgens in een metastase òf in twee verschillende metastasen) - was over het algemeen (in 49 van de 56 gevallen) identiek. Geconstateerd werd dat de ER activiteit tijdens tamoxifen of ethynyloestradiol behandeling daalde tot niet-meetbare waarden, hetgeen inhoudt dat receptor analyses onder deze condities weinig zinvol zijn. Na het staken van deze therapieën dient verscheidene weken gewacht te worden met het nemen van een biopt.

Van alle 388 geanalyseerde tumoren bleek 71% ER-positief. In 68% van de ER-positieve tumoren werd PgR aangetroffen, terwijl het overeenkomstige percentage in ER-negatieve tumoren slechts 9% was. Er bleek een positieve relatie te bestaan tussen het percentage PgR-positieve tumoren en de hoogte van de ER concentratie.

Deze waarnemingen suggereren dat in humane mammacarcinomen, evenals in normale doelwitcellen en in DMBA-geinduceerde mammatumoren, de PgR synthese onder contrôle van oestradiol staat. Bovendien bleek, dat in primaire tumoren vaker PgR werd aangetroffen dan in metastasen. Ook werd vastgesteld dat in 72% van de ER-positieve primaire tumoren PgR aanwezig was, terwijl slechts 55% van de ER-positieve metastasen PgR bevatten. Deze bevindingen duiden erop dat endocriene regulatie systemen verloren gaan wanneer tumorcellen dedifferentiëren. De waarneming dat de PgR activiteit in enkele patienten daalde tot niet-meetbare waarden tijdens het proces van tumor metastasering is hiermee in overeenstemming.

Deze waarnemingen en beschouwingen maken het aannemelijk dat het meten van receptoren voor progesteron naast die voor oestradiol een bijdrage kan leveren aan de selectie van hormoongevoelige tumoren. Uit een recente publicatie (McGuire, 1978), waarin de klinische gegevens van verschillende onderzoekers zijn verzameld, blijkt dat 74% van de patienten met tumoren, waarin beide receptoren aanwezig zijn, gunstig reageerden op endocriene maatregelen. Wanneer wordt afgezien van de aan- of afwezigheid van PgR blijkt slechts 53% van de patienten met ER-positieve tumoren een objectieve remissie te vertonen.

Uit de klinische follow-up van een zeer beperkte groep van patienten (zie proefschrift Beex, 1979) behandeld in het Radboud Ziekenhuis blijkt, dat objectieve remissies bij patienten met ER+ PgR+ tumoren vaker voorkomen dan bij patienten met ER+ PgR- tumoren. Het onderzoek hiernaar wordt voortgezet.

Bij deze wil ik al diegenen bedanken die bijgedragen hebben aan de tot standkoming van dit proefschrift.

In het bijzonder wil ik mijn grote waardering uitspreken voor Anneke Geurts-Moespot, die met grote inzet en enthousiasme op voortreffelijke wijze de meeste experimenten heeft uitgevoerd en meedacht over de interpretatie van de resultaten.

Verder bedank ik Wilma Casimiri-Aalbers, Tineke Corstens en Monique Vossen voor de sympathieke wijze waarop ze een aantal receptor bepalingen voor hun rekening namen.

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Verder bedank ik al diegenen, die voor de juiste verzameling van het patienten-materiaal zorggedragen hebben en vooral alle medewerkers van de Inwendige Geneeskunde (afdeling Endocrinologie, Hoofd Prof.Dr. P.W.C. Kloppenborg, Radboud Ziekenhuis), Chirurgie en Pathologische Anatomie van het Radboud en Canisius Ziekenhuis (met name internist H.S. Tan, Canisius Ziekenhuis).

Tenslotte wil ik Anneke Siebring bedanken voor de attente verzorging van de lay-out en het typewerk van het manuscript.

De schrijver van dit proefschrift werd 12 mei 1950 te Wisch geboren. Hij behaalde het diploma H.B.S.-B aan het Sint Ludger College te Doetinchem in 1968 en begon in datzelfde jaar met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S 2) werd afgelegd in 1971 en het doctoraalexamen met als hoofdvak biochemie (Dr. J.H. Veerkamp) en als bijvakken organische chemie (Dr. W.H. Laarhoven) en experimentele endocrinologie (Dr. Th.J. Benraad) werd behaald in 1974.

Het in dit proefschrift beschreven onderzoek werd medio 1975 gestart, nadat vanuit de universitaire onderzoekpool een formatieplaats voor een wetenschappelijk medewerker werd toegekend. Het project werd uitgevoerd binnen de afdeling Medische Biologie onder leiding van Dr. Th.J. Benraad. Na de promotiedatum zal de schrijver aan een naverwant onderwerp binnen dezelfde afdeling verder kunnen werken dankzij een subsidie van de zijde van het Koningin Wilhelmina Fonds.

STELLINGEN

I

Bij beoordeling van het resultaat van steroid hormoon receptor metingen dient rekening gehouden te worden met de eiwitconcentratie van het geanalyseerde cytosol.

Dit proefschrift, hoofdstuk 2.

II

Vergelijking van meetresultaten van steroid hormoon receptor bepalingen, uitgevoerd binnen verschillende laboratoria is ten zeerste gewenst.

King, R.J.B., Barnes, D.M., Hawkins, R.A., Leake, R.E., Maynard, P.V. and Roberts, M.M. (1978): Br. J. Cancer 38, 428.

Koenders, A.J.M. e.a. (ongepubliceerde waarnemingen) 1979 .

III

Behandeling van het mammacarcinoom middels radiotherapie heeft geen invloed op de oestradiolreceptor status van biopten uit bestraalde tumorlocalisaties.

Le Page, B., Pujol, H., Romieu, Cl., Rochefort, H., Bressot, N. and Saussol, J. (1978). In: Hormone Deprivation in Breast Cancer edited by M. Mayer, S. Saez and B.A. Stoll, p.57. Published by ICI.

Burke, R.E., Mira, J.G., Datta, R., Zava, D.T. and McGuire, W.L. (1978): Cancer Research 38, 2813.

Beex, L.V.A.M. e.a. (ongepubliceerde waarnemingen) 1979.

IV

De correctie voor de aspecifieke binding bij steroid hormoon receptor metingen is slechts juist, wanneer deze gebaseerd is op de door Rosenthal beschreven ontleding van bindingsgegevens.

Rosenthal, H. (1967): Analytical Biochemistry 20, 525.

Blondeau, J.P. and Robel, P. (1975): Eur. J. Biochem. 55, 375.

V

Terecht betwijfelen sommige onderzoekers of bij hormonale behandeling van patienten met een gemetastaseerd mammacarcinoom gestreefd moet worden naar een volledige teruggang van tumorweefsel.

Foulds, L. (1969). Neoplastic Development, vol. 1, p. 73. Academic Press, New York.

Noble, R.L. (1977): Cancer Research 37, 82.

Bruchovsky, N., Rennie, P.S., van Doorn, E. and Noble, R.L. (1978): J. of Toxicology and Environmental Health 4, 391.

VI

De fysiologische betekenis van de zogenaamde zout-resistente vorm van de oestradiolreceptor in de celkern is minder duidelijk dan door een aantal onderzoekers wordt gesuggereerd.

Muller, R.E., Meader, J.E. and Wotiz, H.H. (1978): Cancer Research 38,4233.

Clark, J.H. and Peck, E.J. (1976): Nature 260, 635.

VII

De conclusie van Crain e.a., dat amino fosfolipiden asymmetrisch zijn gelocaliseerd in membranen van staafjes buitensegmenten, wordt onvoldoende gesteund door hun experimentele gegevens.

Crain, R.C., Marinetti, G.V. and O'Brien, D.F. (1978): Biochemistry 17,4186.

VIII

De door Ross beschreven "dialysis rate method" ter bepaling van vrije ioodthyronine en steroid hormonen in bloed verdient in vele gevallen de voorkeur boven de conventionele evenwichtsdialyse.

Ross, H.A. (1978): Experientia 34, 538.

IX

Het valt te verwachten dat de opvallende toeloop van kinderen naar het buitengewoon onderwijs zal afnemen, indien de opleiding van leerkrachten aan de pedagogische academie meer gericht zal zijn op het onderwijs aan en de opvoeding van kinderen met leer-en opvoedingsproblemen.

Bij de interpretatie van gegevens verkregen uit glucagon metingen met behulp van radioimmunoassays wordt onvoldoende aandacht geschonken aan de, reeds enige jaren onderkende, heterogeniteit van plasma glucagon.

Valverde, I., Villanueva, M.L. Lozano, I and Marco, J. (1974): J. Clin. Endocr. and Metab. 27, 1020.

Unger, R.H. (1978): Metabolism 27, 1691.

XI

Bij de discussie over het abortusvraagstuk in de publiciteitsmedia wordt te weinig rekening gehouden met de gevoelens van echtparen, die ongewild kinderloos zijn.

XII

In perioden waarin de vrouw naar emancipatie streeft lijkt haar kleding op die van de man.

A.J.M. Koenders

Nijmegen, 23 mei 1979